Aggregation of Human Immunodeficiency Virus Type 1 by Human Salivary Secretions

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ABSTRACT: Human immunodeficiency virus (HIV-1) is generally transmitted by parenteral contact with infected body secretions. Although extensive epidemiological data and familial studies have failed to provide any conclusive data that saliva may act as a vehicle for transmission of AIDS, both professional and public anxieties remain. The present study, as well as others, suggests that salivary secretions may act as inhibitors of HIV-1 replication in vitro. In our study, the inhibitory activity was determined to be associated mainly with secretions obtained from individuals with the human submandibular-sublingual glands. Human submandibular-sublingual (HSMSL) and parotid (HPS) salivas were collected and tested for their ability to modulate the replication of HIV-1, using a plaque assay on HeLa/CD4+ cell monolayers. Initial results examining freshly collected salivary samples from ten individuals confirmed the results previously obtained by Fox et al. (1988, 1989). An average plaque reduction of ~66% was obtained with HSMSL, in contrast to 34% reduction obtained with HPS. Titration of the inhibitory activity in HSMSL showed detectable levels at a 1:500 dilution. Comparison of inhibitory activity of dialyzed and lyophilized saliva to fresh saliva indicated little difference between the two samples when filtration occurred after the addition of HIV-1. However, the effect of filtration was significantly diminished in the lyophilized samples. Electron microscopic examination of the saliva-HIV incubates revealed the aggregation/entrapment of virus particles by salivary components. These results suggest that human salivary secretions (with HSMSL > HPS) may have a role in modulating the infectivity of HIV-1.

KEY WORDS: saliva, aggregation, HIV, inhibition, and virus.

INTRODUCTION

The human immunodeficiency virus (HIV-1) has been identified as the etiologic agent of acquired immunodeficiency syndrome (AIDS). AIDS was originally classified as a sexually transmitted disease; now, however, the prevalent mode of transmission involves direct parenteral contact with infected body secretions. The presence of infectious virus has been demonstrated in blood and blood products, semen, urine, and, infrequently, saliva (Ho et al., 1985; Groopman et al., 1984). Symptomatic HIV-1-positive individuals frequently have oral mucosal and gingival lesions such as gingivitis and candidiasis, which could increase bleeding into the cavity, thereby increasing the probability of releasing HIV-1 particles into saliva. Epidemiological data and intensive familial studies have failed to provide any conclusive data that saliva may act as a vehicle for transmission of AIDS (Klein et al., 1988; Mann et al., 1986; Friedland et al., 1985). Because of these observations, it has been postulated that salivary secretions may possess the ability to neutralize infectious HIV-1 particles.

A number of studies have indicated that saliva may inhibit HIV infectivity. Fultz (1986) demonstrated that whole saliva has the capacity to inhibit the in vitro replication of HIV in phytohemagglutinin-stimulated peripheral blood lymphocytes. This inhibition was demonstrated by incubating HIV particles with whole saliva, followed by filtration prior to assay. Inhibition of HIV infection was measured as a reduction of the level of reverse transcriptase activity. It was shown that filtration of the saliva before addition of the virus eliminated this inhibitory effect. Fox et al. (1988, 1989) presented further evidence for the direct inhibition of HIV-1 by whole saliva. Samples of saliva, collected from a normal population as well as a group of HIV-seropositive individuals, were incubated with infectious virus
prior to addition to cultured lymphocytes. Their results revealed that whole, unfiltered saliva was capable of inhibiting the in vitro infectivity of HIV-1 as determined by measurement of the levels of the viral reverse transcriptase. This inhibitory activity was present in the human submandibular-sublingual (HSMSL) secretions of normal as well as seropositive individuals. Subsequently, Archibald and Cole (1990) used a focus-forming assay to investigate the role of human saliva in inhibiting the in vitro infectivity of HIV-1 (both cell-free and cell-associated forms). Their results suggested that the inhibitory activity present in whole saliva, HSMSL, and secretions obtained from the minor salivary glands was sensitive to filtration. Collectively, their preliminary data as well as that presented here suggest the presence of a macromolecular factor in human saliva that may inhibit HIV replication. The purpose of this study was to further assess the ability of human salivary secretions to inhibit the in vitro replication of HIV-1 and determine if this activity is the result of aggregation/entrapment of virus particles.

**MATERIALS AND METHODS**

**Collection and Handling of Human Saliva.** Salivas were donated by healthy individuals ages 26 to 39 who were not on any medication and had abstained from eating for at least 2 h prior to collection. Salivary flow was stimulated by applying 2% citric acid to the tongue at 30 s intervals. HSMSL was collected using a custom-fitted collector (Block and Brotman, 1962), whereas parotid saliva (HPS) was collected using a Carlsten-Crittenden apparatus (Curby, 1953). After discarding the first 1 ml of saliva, approximately 10 ml were collected into chilled tubes containing 2.5 ml of 0.1 M Tris-HCl, pH 7.5, with 2% disodium ethylenediaminetetraacetic acid (EDTA) and 5 mM phenylmethylsulfonyl fluoride (PMSF) to minimize protease and glycosidase degradation. Samples were centrifuged at 12,000 × g at 4°C for 30 min then dialyzed and lyophilized as previously described (Levine et al., 1978). Lyophilized saliva samples were reconstituted in phosphate-buffered saline (PBS; 0.15 M sodium chloride, 0.01 M sodium phosphate, pH 7.2) equivalent to original collection volume for inhibition assays. For experiments requiring freshly collected salivas, EDTA and PMSF were omitted and the salivas frozen immediately. For inhibition assays, thawed and reconstituted saliva samples were diluted 1:5 with RPMI 1640 unless noted otherwise. Protein concentrations in salivary samples were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA.).

**Virus and Cells.** The primary HIV-1 virus stock was a titered supernatant obtained from the H9/HTLV-IIIB cell line. HeLa-CD4+ cells (line 6C; AIDS Research and Reference Program, Rockville, Md.) were maintained in RPMI 1640 containing 10% fetal bovine serum (GIBCO, Grand Island, NY) and cultured at 37°C in 5% CO2. For the plaque assay, 3 to 4-day-old cultures of cells were seeded at a density of 2 × 10^4 cells/well into 96 well plates and incubated for 24 h prior to infection.

**Plaque Assay.** The plaque assays were performed as described by Cheesbro and Wehrly (1988). Monolayers of HeLa-CD4+ cells were inoculated with 20 to 40 infectious units of HIV-1. After 90 min at 37°C, the inoculum was removed, monolayers washed, and fresh medium added. Incubation was continued for 3 to 4 days, after which time the infected monolayers were fixed and stained for infectious centers (plaques). To visualize the HIV plaques, monolayers were fixed with methanol, washed with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl (TBS) and incubated for 90 min with a 1:1000 dilution of human anti-HIV standard (AIDS Research Reference Program Cat #192). After washing with TBS buffer, 1:300 dilution of horseradish peroxidase conjugated goat anti-human IgG (Sigma Chemical Co., St. Louis, MO) was added and incubated for an additional 90 min. Plaques were then visualized with aminothyl carbazol (AEC, Sigma Chemical Co., St. Louis, MO) and counted, using an inverted microscope. For inhibition studies, salivary samples were first incubated with HIV-1 for 1 h at 37°C then filtered through a 0.22 μm filter (cellulose acetate; low protein binding) prior to addition to HeLa-CD4+ cell monolayers. After adsorption for 90 min, the inoculum was removed and monolayers incubated as previously stated.

**Electron Microscopy.** The ability of HSMSL and HPS saliva to entrap HIV-1 particles was examined in vitro, using transmission electron microscopy. To increase the chances of locating HIV-1 particles, tenfold concentrated virus samples were obtained by filtration of the infected cell culture supernatant through Centri/ Por concentrators (300 kDa cutoff; Spectrum Medical Industries Inc., Houston, TX). The concentrated virus particles (in 100 μl RPMI 1640 containing 10% FBS) were added to an equal volume of thawed saliva samples in 0.5-ml microfuge tubes and incubated for 2 h at 37°C. To localize the virus particles, the saliva-virus mixtures were then transferred to wells of a Dot Blot apparatus and filtered through a Zeta-Probe membrane (0.45 μm porosity, Bio-Rad Laboratory, Richmond,
CA). After washing the wells with media, the adherent material was fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and filter dots cut into four identical pieces. The pieces were postfixed in 1% OsO₄ in 0.1 M s-collidine buffer, en-block stained with 1% uranyl acetate in 0.1 M maleate buffer, pH 6.0, dehydrated in a graded series of ethanol, and embedded in Epon mixture. Ultrathin cross sections of the filters were cut on an Ultracut-E ultramicrotome equipped with a DuPont diamond knife and double stained with uranyl acetate and lead citrate. The presence of virus particles entrapped in saliva within the filters was determined and photographed using a Hitachi H-600 transmission electron microscope.

**RESULTS AND DISCUSSION**

In agreement with earlier studies, our data demonstrate the ability of salivary secretions to inhibit HIV-1 replication *in vitro*. Submandibular-sublingual saliva was shown to have the highest inhibitory activity; however, significant levels were also present in some parotid secretions. Table 1 shows that HSMSL inhibitory activity ranged from 44 to 100% with an average of 66.3 ± 17.5%, whereas HPS from the same individuals averaged 34.7 ± 29.3%. These data support the previous observations of Fox *et al.* (1988, 1989) that HSMSL possesses the majority of the HIV inhibitory activity.

Titration of the HIV-1 inhibitory activity in HSMSL is presented in Figure 1. Significant levels of inhibitory activity were detected at a 1:500 dilution of freshly collected HSMSL. This level was higher than that detected by Fox *et al.* (1989) or Archibald and Cole (1990). This could be related to the physical number of infectious particles used in each type of assay. The present assay uses severalfold fewer virus particles than do the previous two assays, which could account for the increase in the inhibitory titer of HSMSL. Similar to the studies of Archibald and Cole (1991), the plaque assay used in this study assesses inhibition through the visualization of syncytia. However, the present assay differs in that the target cells were monolayers of HeLa-CD4+ and syncyta visualized using enzyme-linked immunological techniques. The ability to use monolayers enables the visualization of a single infectious center, resulting in the ability to quantitatively assess data from inhibition assays.

The previous studies did not address the mechanism(s) by which saliva inhibits HIV replication. The fact that filtration reduces salivary HIV inhibitory activity indicates that it is possible that aggregation and/or entrainment of virus particles may be one possible mechanism. The data presented in Table 2, showing the effect on inhibition of prefiltration and postfiltration of HIV-1 after mixing with saliva, are consistent with this mechanism. Whole as well as glandular salivas have been shown to form precipitates on freezing (Soderling, 1989). It has been suggested by Dawes (1963) that the precipitate is a result of Ca-proteinates formed following freeze/thawing the saliva. Treatment with disodium EDTA was found to prevent precipitation. The present procedure for lyophilization of HSMSL and HPS involves treatment with EDTA during the collection and initial dialysis. This should result in a reduction in the formation of Ca-proteinates during the reconstitution of lyophilized HSMSL. The data obtained from fresh HSMSL (Table 2) demonstrate a significant loss (83 vs. 14%) of the inhibitory activity when saliva was filtered prior to addition of HIV. This loss appears to correlate with the loss of particulate material in the saliva sample. In the lyophilized sample of HSMSL, a detectable loss of activity was also seen but it was not as great as with the fresh sample. This result appears to correlate with the decreased amount of particulates present in the reconstituted sample.

**TABLE 1**

**Inhibition of HIV-1 Infectivity by Freshly Collected Salivary Secretions**

<table>
<thead>
<tr>
<th>Percent Inhibition by</th>
<th>Donor</th>
<th>HSMSL</th>
<th>HPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>83</td>
<td>40</td>
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</tr>
<tr>
<td>4</td>
<td>100</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>59</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>65</td>
<td>80</td>
<td></td>
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<tr>
<td>8</td>
<td>50</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>65</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

*Note:* Samples of HSMSL and HPS collected from each individual were diluted 1:5 with RPMI 1640 prior to assay. The percent reduction of pfu (plaque-forming units) was determined for each sample as performed in triplicate.
FIGURE 1. Titration of HIV-1 inhibitory activity present in human sub-mandibular-sublingual saliva. Dilutions of fresh HSMSL (in quadruplicate) were incubated with HIV-1-infected cell supernatant for 90 min at 37°C and the reduction in the number of plaques determined as described.

TABLE 2
Effect of Filtration on Inhibitory Activity in Fresh and Lyophilized HSMSL and HPS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mg/ml protein)</th>
<th>Prefiltration % inhibition of pfu</th>
<th>Postfiltration % inhibition of pfu</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSMSL (lyophilized)</td>
<td>1.5</td>
<td>84 ± 17</td>
<td>51 ± 11</td>
</tr>
<tr>
<td>HPS (lyophilized)</td>
<td>0.3</td>
<td>74 ± 8</td>
<td>34 ± 13</td>
</tr>
<tr>
<td>HSMSL (fresh)</td>
<td>2.6</td>
<td>43 ± 13</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>HPS (fresh)</td>
<td>0.42</td>
<td>22 ± 11</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>HSMSL (fresh)</td>
<td>0.7</td>
<td>83 ± 12</td>
<td>14 ± 9</td>
</tr>
<tr>
<td>HPS (fresh)</td>
<td>1.1</td>
<td>36 ± 9</td>
<td>38 ± 4</td>
</tr>
</tbody>
</table>

Note: In prefiltration, samples of HSMSL and HPS were mixed with HIV-1, incubated, and then filtered. For postfiltration, samples of HSMSL and HPS were filtered prior to addition of virus. Fresh saliva samples were diluted 1:1 with 2 x PBS prior to assay. Lyophilized samples were tested at undiluted and at a 1:5 dilution. The reduction in pfu (plaque forming units) is expressed as the average of four assays performed in triplicate ± standard deviation.
lected on Zeta-probe membranes. After preparation of the membranes for electron microscopy, each membrane dot was cut into four equal quadrants from which five to eight thin cross sections from each were prepared and examined for HIV-1 particles entrapped in salivary particulates. At the ultrastructural level, clear morphological differences between the filter retentates of HPS and HSMSL were observed. HPS displayed moderately electron-dense material, consisting of primarily fine granules with a homogeneous size, and tended to form multilamellated configurations (Figure 2a) in the pores of the filter. In contrast, HSMSL saliva appeared as amorphous materials that were composed of electron-dense granular and filamentous components of various sizes (Figures 2b,c). As can be observed in Figure 2, transmission electron microscopy revealed the presence of a group of virus particles that were either embedded in (Figure 2b), or associated with (Figure 2c), precipitate obtained from HSMSL. These particles demonstrated a morphology typical of mature HIV-1. They had a diameter of 100 to 120 nm and contained the dense core structure and the envelope characteristic of HIV-1 (Smith et al., 1990; Meyenhofer et al., 1987; Epstein et al., 1985). However, no virus particles were observed in the retentate of HPS (Figure 2). These data strongly suggest that one mechanism responsible for the reduction of HIV-1 infectivity after treatment with saliva results from entrapment and/or aggregation of virus particles by salivary secretions.

Although the presence of this aggregating ability would account for the reduction of the number of plaques in this assay, other mechanisms for inhibition cannot be ruled out. Clements et al. (1991) has sug-

FIGURE 2. Electron micrograph of human parotid and submandibular-sublingual salivas incubated with HIV-1. Saliva samples were incubated with tenfold concentrated HIV-1-infected culture supernatant. The saliva/HIV mixture was filtered through a membrane that was then sectioned for EM. (a) HPS incubated with HIV-1 (magnification x 65,000). (b, c) HSMSL incubated with HIV-1, (magnification x 170,000 and 240,000, respectively). S: salivary component; F: filter; E: HIV-1 envelope; arrowheads: HIV-1 core.
gested that proteolysis of HIV-1 surface glycoproteins inhibits virus replication. Human whole saliva contains a number of proteolytic enzymes, most of which are of microbial origin (Nakamura and Slots, 1983). The presence of proteolytic enzymes in glandular secretions has also been determined (Makinen, 1989). More recently, studies have indicated that sulfated monosaccharides and polysaccharides interfere with adsorption/penetration of HIV-1 as well as syncytia formation (Bagasra et al., 1991). Anionic salivary glycoproteins (e.g., salivary mucins) in HSMSL have been shown to contain, in addition to N-acetyl-neuraminic acid, covalently linked sulfate as N-acetylgalactosamine-4-sulfate (Levine et al., 1985).

In conclusion, this study confirmed previous results showing a HIV-1 inhibitory activity in salivary secretions. In addition, we have presented direct evidence for the entrapment of virus particles by precipitated salivary components as one possible mechanism for this inhibition. These precipitates are heterotypic complexes that have been shown to contain a number of salivary proteins and glycoproteins. These include, salivary mucins, secretory IgA, lysozyme, peroxidase, amylase, statherin, and detectable amounts of the proline-rich proteins (M.S. Reddy, personal communication; Tabak et al., 1982). Components present in these heterotypic complexes will next be isolated and tested for inhibitory activity. Further studies will be required to assess the possible role of other salivary components in the inhibition of HIV-1 replication.

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