Human Submandibular Saliva Inhibits Human Immunodeficiency Virus Type 1 Infection by Displacing Envelope Glycoprotein gp120 from the Virus

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Human submandibular saliva reduces human immunodeficiency virus type 1 (HIV-1) infection in vitro. To define the mechanism of inhibition, virus was incubated with saliva or medium, velocity sucrose gradient centrifugation was performed, and fractions were analyzed for p24 and gp120. The results show that after incubation with saliva, the envelope glycoprotein was displaced from both a laboratory-adapted and a low-passage clinical HIV-1 isolate. To identify the salivary protein(s) responsible, submandibular saliva was fractionated by anion-exchange chromatography. Protein fractions containing anti-HIV activity were assayed for their ability to strip gp120 from virus. The partially purified active fractions contained two high-molecular-weight sialyated glycoproteins identified as salivary agglutinin and mucin, as well as several lower-molecular-weight proteins. It thus appears that specific salivary proteins interact with HIV-1 to strip gp120 from the virus with a resultant decrease in infectivity.

Fultz [1] demonstrated that incubation of human immunodeficiency virus type 1 (HIV-1) with human or chimpanzee saliva inhibits infection of peripheral blood mononuclear cells (PBMC). Several groups demonstrated that incubation of virus with saliva causes virus aggregation, as demonstrated by electron microscopy, and subsequent loss of virus titer upon filtration [2–7]. Salivary mucin was suggested to mediate this activity [6], which is found in both whole saliva and ductal saliva, with the highest activity observed in submandibular saliva [3]. We showed that saliva obtained from many seronegative individuals exhibits anti-HIV activity in the absence of a filtration step [8] and that the inhibitory activity is specific to HIV-1, with little or no activity against adenovirus, herpes simplex virus type 1 (HSV-1), HIV-2, and simian immunodeficiency virus [3, 8]. McNeely et al. identified a salivary protein that reduces the susceptibility of monocytes, macrophages, and CD4+ T cells to HIV and determined that the protein was a secretory leukocyte protease inhibitor [9]. Crombie et al. [10] attributed anti-HIV activity in saliva to another protein, thrombospondin 1 (TSP1). Therefore, saliva appears to inhibit HIV infection by several different mechanisms.

While epidemiologic studies suggest that oral transmission of HIV is rare [11, 12], several reports have raised the possibility of such transmission. Baba et al. [13] reported productive infection of macaques after application of simian immunodeficiency virus to the back of the tongue, and Schacker et al. [14] studied high-risk individuals and identified oral sex as the sole risk factor in 4 subjects who seroconverted. While 50%–80% of saliva samples obtained from HIV-seropositive persons were positive for HIV sequences by RNA or DNA amplification techniques [15, 16], isolation of infectious virus from oral samples is rare [2, 17–22]. This suggests that salivary anti-HIV factors may serve to block infectivity in vivo.

This report further investigates mechanisms whereby submandibular saliva inhibits HIV-1 infectivity in the absence of filtration. Previously we showed that the inhibitory activity cannot be explained by the effect of saliva on cells and that saliva does not lyse virus [23]. Here we investigated whether incubation of saliva with purified virus leads to release of gp120 from the virion and whether that release correlates with loss of infectivity after exposure of virus to salivary proteins.

Materials and Methods

Viruses, cells, and saliva. Virus stocks were prepared in PBMC that were obtained from healthy HIV-1–seronegative donors. PBMC were isolated by Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient centrifugation and grown in RPMI containing 10% fetal calf serum, gentamicin (10 μg/mL), and phytohemagglutinin (2 μg/mL) for 48 h. Interleukin (IL)-2 (10 units/mL) was added for 48–72 h, and then cells were infected with HIV-1. Viruses studied included laboratory-adapted HIV-1_LAI and a low-passage HIV-1 isolate (92US076, syncytial, postpartum infant isolate) obtained from the AIDS Research Reference Reagent Program (NIH, Bethesda, MD). To determine whether the low-passage isolate was M- or T-
tropic, infection was performed in U373-MAGI, U373-MAGI-CCR5E, and U373-MAGI-CXCR4CEM cell lines [24].

Human submandibular saliva was obtained from HIV-seronegative donors using a plastic collector that was constructed for each donor to fit over the lower jaw and to collect saliva directly from the submandibular duct located in the floor of the mouth. After collection, saliva was pooled, dialyzed, and lyophilized, as previously described [3, 8].

**Virus purification.** Supernatants of infected PBMC were centrifuged at 3000 g and passed through a 0.45-μm filter to remove cellular debris and then centrifuged at 112,700 g for 2 h at 4°C through a 5% sucrose cushion. The virus pellet was suspended in 1 mL of RPMI and fractionated on a sephacryl 1000 column to remove free gp120 [25]. The column was eluted with PBS, and fractions were tested for infectious virus in PBMC. Fractions containing the highest titers were pooled and stored at −70°C.

**Antibodies.** Monoclonal antibodies to p24 and gp120 were obtained from the AIDS Research Reference Reagent Program. Polyclonal antibody to saliva agglutinin (SAG) was prepared as previously described [26]; anti-mucin (MG2) polyclonal antibody was obtained from Michael Levine (State University of New York at Buffalo).

**Detection of HIV-1 gp120.** Assays were performed to determine if saliva removes gp120 from HIV-1. Saliva or medium was incubated with 250 ng of purified virus that was quantified by measuring p24 antigen content. Virus and medium, virus and saliva, or virus lysed with Triton X-100 (0.1% in PBS) was placed on a 4.5-mL 10%–60% sucrose gradient and centrifuged at 145,000 g for 2.5 h at 4°C. Fractions of 250 μL were collected from the top of the gradient and analyzed for p24 and gp120 by ELISA (NEN-DuPont, Boston).

As an additional assay for measuring the removal of gp120, virus was incubated with medium, saliva, or partially purified salivary protein fractions and centrifuged at 145,000 g for 2.5 h at 4°C onto a 5% sucrose cushion. The pellet was suspended in 100 μL of PBS, and proteins in the supernatants were concentrated by precipitation with 10% trichloroacetic acid (TCA). The pellet and TCA-precipitated supernatants were analyzed by Western blot using antibodies to gp120 and p24 [27] or by ELISA (NEN-DuPont).

**Salivary protein fractionation by anion-exchange chromatography.** Lyophilized saliva was resuspended in 25 mM Tris buffer (pH 8.0) and filtered through a 0.22-μm membrane. The filtrate was placed on a MonoQ column (Pharmacia) and eluted using a 0–0.5 M NaCl linear gradient at 0.5 mL/min. Protein concentration in the fractions was monitored by absorbance at 280 nm. Fractions were concentrated and analyzed on 6% or 10% SDS-PAGE gels that were stained with silver (Bio-Rad, Hercules, CA) or Alcian Blue [28] to visualize proteins or carbohydrates, respectively. Duplicate gels were blotted and reacted with polyclonal antibody to MG2 or SAG.

**Viral infection assays.** HeLa-CD4LTR-βgal cells were used to measure viral infection [29]. Submandibular saliva samples (200 μL) or column fractions were incubated with an equal volume of HIV-lgα (50 ng) for 1 h at 37°C and added to cells for 3 h. Cells were washed with PBS and, after 48 h, were fixed and stained with X-gal. The percent inhibition was calculated by counting the number of blue cells when virus was incubated with saliva or column fractions compared with virus alone.

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**Figure 1.** Velocity sucrose gradient centrifugation of purified virus on 10%–60% sucrose gradient. **A**, Virus was lysed with Triton X-100; **B**, virus was incubated with medium; and **C**, virus was incubated with submandibular saliva. Fractions (n = 20) were collected and analyzed by ELISA for p24 (expressed as pg/5 μL). Alternate fractions were assayed for gp120 and expressed as ng/mL.
Results

**Effect of saliva on virus-associated gp120.** Purified HIV-1$_{1182}$ was incubated with submandibular saliva or as control with medium or Triton X-100 and then placed on a sucrose gradient. When virus was lysed with Triton X-100, p24 and gp120 appeared at the top of the gradient (figure 1A). When virus was incubated with medium, gp120 and p24 fractions comigrated in the gradient (figure 1B). In contrast, when virus was incubated with saliva, gp120 dissociated from the p24 fractions and migrated toward the top of the gradient (figure 1C). Because p24 is a marker for the position of HIV-1 capsid protein in the gradient, these results suggest that saliva removes gp120 from the virus.

To confirm this observation and to simplify the assay procedure, purified HIV-1 was incubated with saliva or medium and centrifuged through a 5% sucrose cushion. Supernatant and pellet were separated and assayed by Western blot for p24 and gp120 (figure 2). Supernatants were concentrated by TCA precipitation before electrophoresis (figure 2). When virus was incubated with medium, both gp120 and p24 remained associated with the virus and appeared in the pellet. In contrast, when virus was incubated with saliva, gp120 appeared in the supernatant and was displaced from the virus, as measured by p24 antigen.

**Anion-exchange chromatography of submandibular saliva.** To identify the protein(s) involved in saliva-associated anti-HIV activity, submandibular saliva was fractionated by anion-exchange chromatography on a MonoQ column (figure 3A). Fractions were evaluated for anti-HIV activity using HeLa-CD4 cells that measure HIV LTR-driven β-galactosidase expression. The anti-HIV activity was observed in fractions 7–9 (figure 3B). Anion-exchange fractions were collected, run on SDS-PAGE gels, and stained with Alcian Blue–silver, which revealed two prominent bands that were most intense in fractions 8 and 9. This included a darker staining band of ~130 kDa and a fainter staining band of ~350 KDa (figure 3C). Western blotting identified the higher-molecular-weight protein as SAG (figure 3D) and the 130 KDa as low-molecular-weight mucin (MG2) (figure 3E). In addition, these fractions contained several lower-molecular-weight proteins seen on silver staining (figure 3F).

**Effect of saliva and partially purified salivary proteins on a primary HIV-1 isolate.** Laboratory-adapted HIV-1 isolates more readily dissociate gp120 than do low-passage clinical isolates [30]; therefore, we evaluated whether saliva affected HIV-1 92US076, a low-passage clinical isolate. We determined the M- or T-tropic phenotype of the low-passage isolate by meas-

![Figure 2](image)

**Figure 2.** Western blot analysis of purified virus incubated with saliva. Purified HIV-1$_{1182}$ was incubated with medium or saliva and centrifuged onto 5% sucrose cushion. Virus pellet and trichloroacetic acid–precipitated supernatants were analyzed by Western blot for p24 and gp120 antigen. A, gp120 in pellet (lanes 1 and 2) and supernatant (lanes 3 and 4). B, p24 antigen in pellet (lanes 1 and 2) and supernatant (lanes 3 and 4).

![Figure 3](image)

**Figure 3.** Anion-exchange fractionation of human submandibular saliva. A, Submandibular saliva was run on MonoQ column and eluted using linear gradient of 0–0.5 M NaCl. Protein profile (A$_{280}$ nm) is shown. B, Fractions (1–11) were dialyzed and analyzed for anti-HIV activity. Percent inhibition was calculated by comparing virus incubated with salivary fractions with virus incubated with medium alone and expressed as average % inhibition for triplicate determinations.
Figure 3. Continued. Anion-exchange fractionation of human submandibular saliva. C–F, Column fractions were analyzed by SDS-PAGE. In C–E, aliquots were electrophoresed in 8% SDS gels, stained for carbohydrate with Alcian Blue–silver (C), or blotted and reacted with polyclonal antibody to salivary agglutinin (SAG) (D) or anti-mucin (MG2) (E). In F, fractions were concentrated 10×, run on 10% SDS gel, and silver-stained to show protein pattern.
Figure 4. Stripping of gp120 from low-passage clinical isolate. Submandibular saliva, fractions 7–9, or culture medium were incubated with purified primary HIV isolate. After centrifugation, pellet was tested for gp120 and p24 by Western blot. Radiograph was scanned by densitometry, and ratio of gp120 to p24 was determined to compare virion-associated gp120 in samples treated with medium, saliva, or partially purified salivary proteins (fractions 7–9). Position of gp120 and p24 are shown on left.

Since the amount of purified primary isolate loaded was less compared with HIV-1, the supernatant collected after the centrifugation contained insufficient gp120 to detect by Western blot. Therefore, we quantified gp120 in supernatants by ELISA. When virus was incubated with medium as control, 16% of the total gp120 appeared in the supernatant compared with 58% and 52% for saliva and partially purified saliva in fractions 7–9, respectively (figure 5). Therefore, saliva and partially purified salivary proteins displaced gp120 from both a laboratory-adapted and a low-passage clinical HIV-1 isolate.

Discussion

We studied the effect of submandibular saliva and salivary fractions obtained by anion-exchange column chromatography on a laboratory-adapted and a primary HIV-1 isolate. We found that submandibular saliva dissociates gp120 from the virion, indicating that the anti-HIV activity of submandibular saliva results from a direct effect of saliva on the virus. This is consistent with our previous results, which showed that preincubation of target cells with saliva did not block HIV-1 infection [23]. The viruses studied were T-tropic; therefore, it will be of interest in future studies to evaluate M-tropic viruses to determine if saliva is also active against these isolates. The inhibitory factor(s) we are defining differ from those of McNeeley et al. [9], who reported that anti-HIV activity in human saliva was associated with secretory leukocyte protease inhibitor, which reduces viral infection apparently by interacting with surface protein(s) on host cells [31]. Turpin et al. [32], however, questioned these findings.

Bergey et al. [6] showed that purified salivary MG2 inhibits HIV infection of HeLa-CD4 cells, and that this inhibition is...
associated with viral aggregation. We fractionated submandibular saliva by anion-exchange chromatography and demonstrated that these salivary glycoproteins inhibit infection by both agglutinating virus and dissociating the envelope glycoprotein. Bergey et al. [6] showed that purified MG2 interacts with gp120, which supports a possible role for MG2 in dissociating gp120. Our studies suggest that SAG may be more active than MG2, on the basis of the finding that fraction 10 in figure 3B showed no HIV-1 inhibitory activity yet contained MG2 (figure 3E). However, the two glycoproteins might work together, and quantities below the level of detection on Western blot may be sufficient for HIV-1 inhibition; therefore, dose-response studies with purified SAG and MG2 used alone and in combination will be required to define the relative contribution of each glycoprotein.

It has previously been shown that incubation of virus with soluble CD4 or anti-gp120 antibodies removes gp120 from the virion [25, 30, 33]. These molecules do not likely account for our results because there is no evidence of a soluble CD4-like molecule in saliva [7], and saliva was obtained from HIV-seronegative donors. Recent studies suggest that TSP1 is found in submandibular saliva and binds to gp120 to inhibit viral infectivity [10]. TSP1 binds to proteins that share sequence homology with the C2 and C3 domains of gp120 that adjoin the V3 loop. These regions are involved in gp120 binding to CD4. Therefore, it was suggested that TSP1 may inhibit HIV-1 infection by preventing gp120-CD4 interaction. TSP1 is a higher-molecular-weight molecule than MG2 or SAG, which are the most abundant molecules detected in biologically active salivary fractions 7–9. In addition, the salivary activity we report does not block gp120-CD4 interaction (unpublished observation). Nevertheless, further studies are required to determine whether TSP1 accounts for any of the anti–HIV-1 activity noted in our report.

Archibald et al. [34] showed that salivary proteins bind to gp120 sequences and reduce binding of monoclonal antibodies. It is possible that the interaction of salivary proteins with gp120 might result in conformational changes that weaken the association of gp120 with gp41. It is also possible that salivary proteins bind to regions of gp41 and distort its interaction with gp120 or enzymatically remove portions of gp120. We showed previously that the anti-HIV activity of saliva appears not to be associated with proteolytic activity [35]. Therefore, our current hypothesis is that salivary proteins bind to specific regions on gp120 and that binding alters the hydrophobic association between gp120 and gp41, resulting in gp120 shedding. It will be of interest to determine whether similar HIV-1 inhibitory factors are present in cervical secretions, semen, or parotid saliva. Future studies using purified proteins will address whether MG2, SAG, or both have anti-HIV stripping activity, or whether this activity requires the lower-molecular-weight proteins detected in fractions 7–9.

Acknowledgments

We thank Michele Kim for technical assistance and the NIH AIDS Research Reference Reagent Program for monoclonal antibodies to p24 and gp120, for the HIV-1 primary isolate, and for U373-MAGI, U373-MAGI-CXCR4CEM, and U373-MAGI-CCR5E cell lines.

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