Oral Transmission of Human Immunodeficiency Virus by Infected Seminal Fluid and Milk: A Novel Mechanism

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Salivary transmission by the 30 million human immunodeficiency virus (HIV) carriers is rare, despite kissing, aerosolization, and dental treatment. The main protective mechanism of saliva is reported to be inactivation of HIV-transmitting leukocytes by its unique hypotonicity; however, the successful oral transmission of HIV by seminal fluid and milk is unexplained. Whether seminal fluid and milk successfully transmit HIV orally by overcoming the recipient’s salivary hypotonic inactivation of HIV-transmitting leukocytes was tested. Isotonic salt solution and normal donor samples of milk, colostrum, seminal fluid, and blood were studied for their ability to overcome the salivary hypotonic inactivation. All samples, in physiologic volumes, prevented the hypotonic saliva from inactivating HIV-transmitting leukocytes by providing solutes and retarding diffusion. This indicates that successful oral transmission of HIV by seminal fluid, milk, and colostrum may be due to their isotonicity, which overcomes hypotonic salivary inactivation of HIV-transmitting leukocytes.

Materials and Methods

Specimens. Six samples of milk were collected from normal donors 3–4 days after delivery. Three samples of colostrum were collected from normal donors 0–2 days after delivery. Samples of saliva were collected from 6 normal donors and were centrifuged and filtered through a 0.22-μm filter for sterilization. Seminal fluids were collected from 3 normal men. Samples were stored at 4°C for 1–2 days or, alternatively, stored at −20°C for up to 3 months. This storage was determined not to affect the action of seminal fluid, milk, or colostrum. Blood was obtained from 2 normal donors and was used immediately.

Viruses. Stocks of the 213 strain of HIV were propagated in human H9 lymphocytes by means of standard procedures [40]. A second HIV strain, AC-1, is equally well inhibited by saliva-treated peripheral blood mononuclear leukocytes (PBL) [9]. Aliquots were stored frozen at −70°C. The Indiana strain of vesicular stomatitis virus (VSV) was propagated in murine L cells, as described elsewhere [41]. VSV stocks were stored frozen at −70°C.

Cells. Human PBL and the human lymphocyte cell line CEM were prepared and propagated in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics, as described elsewhere [9]. Human peripheral blood macrophages were obtained by ficoll-hypaque purification of normal PBL [9]. To obtain macrophages, the purified PBL were allowed to adhere to a glass surface for 3 h at 37°C. Unattached mononuclear leukocytes were rinsed away with medium, and the adherent macrophages were used in the experiments of cell viability in the presence of saliva.

Virus multiplication. The effects of the various treatments on virus multiplication were determined in virus-infected human CEM lymphocytes, PBL, or L929 cells. The multiplication of HIV in PBL was determined as the yield of infectious HIV from 2 × 10^7 ficoll-hypaque-purified normal PBL that had been cultured with phytohemagglutinin (4 μg/mL) for 2 days before treatment with 20 U/mL interleukin (IL)-2 (40 U/mL) for an additional 2 days and then infected with 10^2 TCID_50 of HIV strain 213. The infected cells were then incubated with 20 U/mL IL-2 for 5 days, washed 4 times, and incubated with undiluted saliva, the various mixtures, or culture medium for 15–60 min. These leukocytes were subsequently washed and cultured for HIV production in RPMI 1640 tissue culture medium plus 15% fetal bovine serum and 20 U/mL IL-2 for 24 h before the cell-free medium was harvested for assay of...
HIV yield, as described below. In experiments that used the human CEM lymphocyte cell line [42], multiplication was determined as the yield of infectious HIV from $6 \times 10^5$ CEM lymphocytes that were infected with $10^2$ TCID$_{50}$ of HIV strain 213. The cells were then incubated for 4 days and washed 4 times before 15- to 60-min treatment with the various preparations. These infected lymphocytes were subsequently washed 4 times and cultured for 24 h before being harvested for assay of HIV production, as described below. For experiments requiring noncontainment facilities in which to perform more complex procedures, we substituted a safer surrogate virus, vesicular stomatitis virus (VSV), in place of HIV. Other surrogate viruses have been used previously [9]. VSV multiplication was measured, similarly, as the yield of infectious virus in the culture medium from the CEM lymphocytes or murine L cells [9]. This was done after infection with 3000 pfu of virus.

Pilot experiments were done initially with VSV-infected human CEM lymphocytes or murine L cells. Confirmatory experiments were done with HIV-infected CEM lymphocytes and PBL [9]. All studies were replicated 3 times. Assays of HIV and VSV production. The production of HIV in the experiments was determined by the standard TCID$_{50}$ assay with MT-2 human lymphocytes [42]. Specifically, culture fluids harvested from the HIV-infected human CEM lymphocytes [9] were serially diluted in 0.5-log$_{10}$ increments in RPMI culture medium containing 10% fetal bovine serum. Next, 50 $\mu$L of each dilution was added to quadruplicate microtiter wells, each containing 110 $\mu$L of 2 $\times$ 10$^4$ MT-2 cells in culture medium. After the serial dilutions, 120 $\mu$L of nutrient medium was added to each well. These microtiter plates were incubated at 37°C for 3 days in a CO$_2$ incubator and then were again fed culture medium containing 10% fetal bovine serum. The wells were read for HIV multinucleated giant cell cytopathic effect on day 5 or 6. The TCID$_{50}$ was determined by the method of Reed and Muench [43], and the production of VSV was determined as pfu in L cells [9].

Statistics. In addition to basic descriptive statistics in the figure legends, statistical methods such as regression analysis and non-parametric methods were used, with no adjustments for multiple testings. In each instance, the pertinent trends and differences observed were large, and all related significance levels ($P$ values) were small, even with sample sizes used.

Results

Reversal of Salivary Inactivation of Virus-Infected Leukocytes by Raising the Tonicity of Saliva with Seminal Fluid, Milk, or Isotonic Salt Solution

We hypothesized that reconstitution of salts in hypotonic saliva by orally deposited isotonic seminal fluid, milk, or colostrum would reverse saliva’s ability to inactivate the virus-infected leukocytes contained in those secretions [44–50]. To determine the ratio of saliva to an isotonic salt solution that could prevent cell lysis, we serially diluted saliva in varying increments in isotonic Dulbecco’s balanced salt solution. The experiments were done first with the surrogate VSV (see Methods) and then with HIV. In the first experiments, the dilutions were used to treat VSV-infected L929 cells for 1 h to allow inactivation of cells to occur. The treated cells were then washed and resuspended in medium for 24 h of incubation for virus production, as described above. Because virus can be produced only by surviving viable cells, any decrease in virus yield would indicate inactivation of infected cells by saliva [9]. As shown in figure 1A, strong inhibition of virus production by infected L cells occurred with mixtures of saliva and isotonic balanced salt solution containing 70%–90% saliva, thereby confirming that infected cells were inactivated by high concentrations of saliva and, consequently, did not produce virus. Diminished inhibition of virus yield occurred with the mixtures containing 35%–65% saliva, showing partial inactivation of cells by saliva and thereby indicating partial protection of cells. Insignificant inhibition of virus production occurred with <55% saliva, indicating complete protection of cells from salivary inactivation. These findings were confirmed by use of human PBL. When normally isotonic seminal fluid, milk, or colostrum was substituted for the isotonic salt solution on VSV-infected human CEM lymphocytes, similar reversals of virus yields were found (figure 1B). These findings were confirmed with HIV-infected CEM lymphocytes (figure 1C and 1D). The findings were also confirmed with HIV-infected normal PBL. Thus, dilution of hypotonic saliva with isotonic body fluids such as seminal fluid, milk, and colostrum overcame the protective action of saliva against HIV-infected leukocytes.

Another possibility is that sufficient bleeding into the mouth may contribute enough toxicity to overcome the salivary inactivation of the HIV-infected leukocytes in the shed blood [9]. Consequently, we determined whether dilution of saliva in heparinized blood prevented saliva’s inhibition of HIV multiplication in human CEM lymphocytes infected with HIV. As shown in figure 1E, dilution in blood did prevent saliva’s inhibition of HIV multiplication, with a dilution response curve similar to those obtained by use of seminal fluid, milk, colostrum, and isotonic salt solutions. Controls showed no inhibition of HIV at the concentration of heparin that was used. Thus, heavy bleeding into the mouth may pose a hazard to health care workers and to other contacts. These are a case report of HIV transmission by biting during heavy bleeding into the mouth that supports this possibility [51].

To confirm the findings obtained with the CEM lymphocyte cell line that dilution of hypotonic saliva with isotonic body fluids overcame the protective action of saliva, we repeated the experiments with normal human PBL. The findings confirm for HIV-infected PBL those obtained by use of CEM lymphocytes (figure 1F).

We next calculated whether the volumes of orally deposited seminal fluid, milk, colostrum, or blood were sufficient to overcome salivary protection. Because the residual volume of saliva in the mouth averages 0.75 mL [52], we may estimate that the volume of orally deposited seminal fluid, milk, or colostrum required to partially protect its infected leukocytes against salivary lysis is 0.25–0.45 mL. Complete protection of shed leu-
Figure 1.  

A. Reversal of salivary inhibition of vesicular stomatitis virus (VSV) multiplication in infected L cells. Saliva was diluted in isotonic balanced salt solution in increments shown. Each diluted sample was incubated for 1 h with VSV-infected murine L929 cells in 4 replicate microtiter wells. Cells were then washed 3 times with Hanks’ balanced salt solution, fed Eagle’s basal medium containing 2% fetal bovine serum, and incubated for 18 h before supernatants were harvested to determine virus production, by use of virus plaque assay on L929 cells. Log₁₀ inhibition of VSV yield was calculated by comparing virus yield in control cultures with that in treated cultures.  

B. Reversal of salivary inhibition of VSV multiplication in CEM lymphocytes by dilution in medium, human seminal fluid, or milk. Procedures were as in A except that saliva was diluted in seminal fluid, milk, colostrum, or medium, and VSV-infected human CEM lymphocytes, rather than L929 cells, were treated with samples.  

C and D. Dilution in seminal fluid or milk, respectively, prevents saliva’s inhibition of human immunodeficiency virus (HIV) multiplication in CEM lymphocytes. Procedures were as in B except that HIV-infected CEM lymphocytes were treated with samples of saliva diluted in seminal fluid or milk, respectively.  

E. Dilution in blood prevents saliva’s inhibition of HIV multiplication in normal human peripheral blood leukocytes (PBL). Procedures were as in C except that blood was used instead of milk, and PBL were substituted for CEM cells.  

F. Dilution in blood, milk, colostrum, and seminal fluid prevents saliva’s inhibition of HIV multiplication in human PBL. Procedures were as in C–E. *P < .05 by Student’s t test.

Kocytes would be expected to occur with deposited volumes >0.45 mL (<65% saliva in figure 1B). This prevention of salivary protection should occur normally in the mouth, because the minimum 0.45-mL volume of seminal fluid, milk, or colostrum required for prevention of salivary protection is easily exceeded by the normal 3-mL volume of seminal fluid [53, 54] and also by the >50 mL of deposited milk or colostrum. Blood in excess of 0.45 mL also would prevent salivary inactivation. Thus, the
Figure 2. Human milk protects vesicular stomatitis virus (VSV)-infected CEM lymphocytes exposed to saliva: effect of time of incubation. Procedures were as in figure 1 except that 450 µL of saliva was layered (without mixing) over 50 µL of milk containing $5 \times 10^6$ infected CEM lymphocytes and was incubated for either 15 or 60 min. * by Student’s t test. 

Figure 3. Colostrum protects vesicular stomatitis virus (VSV)-infected CEM lymphocytes against inactivation by saliva: effect of volume of colostrum. Procedures were as in figure 2, with 60 min of incubation, except that volumes of colostrum containing infected CEM lymphocytes were 0.025, 0.1, and 0.5 mL and saliva volume was appropriately increased to maintain 10 : 1 saliva-to-sample proportion. *P < .05 by Student’s t test.

usual volumes of seminal fluid, milk, or colostrum deposited in the mouth are sufficient to overcome saliva’s normal protection of HIV-infected leukocytes and thereby may account for the successful oral transmission by these fluids.

Do Seminal Fluid and Milk Physically Impede Penetration by Saliva’s Hypotonicity? 

Effect of time of incubation with seminal fluid or milk on salivary inactivation of virus-infected leukocytes. To help determine whether seminal fluid and milk protected leukocytes infected with the surrogate VSV, not only with their tonicity but also by physically impeding diffusion of solutes and water, we determined the time required for the hypotonicity to permeate seminal fluid or milk containing infected leukocytes. Saliva (450 µL) was layered over 50 µL of seminal fluid, milk, or colostrum containing $2 \times 10^6$ CEM lymphocytes and then was incubated for 15 or 60 min before being assayed for inactivation of the infected leukocytes. As shown for milk in figure 2, the longer incubation time of 60 min increased inhibition of virus yield, thereby indicating decreased protection of virus-infected cells by longer contact with saliva. Similar results were obtained with seminal fluid. These findings indicate that seminal fluid and milk can delay penetration of salivary water and outward diffusion of solutes from the seminal fluid and milk, thereby partly protecting their virus-infected leukocytes.

Effect of volume of seminal fluid or milk on salivary inactivation of virus-infected leukocytes. Another indicator of diffusion is the effect of droplet size of seminal fluid and milk on the ability of saliva to inactivate infected leukocytes. We expected that leukocytes contained in larger droplets of seminal fluid or milk would be more resistant to inactivation, because their larger radius would delay the diffusion of solutes and water. To test this possibility, we layered saliva over different volumes of seminal fluid or milk while keeping the saliva-to-sample ratio at 10 : 1. In the representative experiment shown in figure 3, the greater the volume of colostrum, the less the inactivation of VSV-infected human CEM lymphocytes by saliva. The findings suggest that larger droplets of seminal fluid, milk, or colostrum could provide greater protection of virus-infected lymphocytes, thereby indicating reduction of diffusion between saliva and these fluids.

Effect of mixing saliva with seminal fluid or milk on inactivation of virus-infected leukocytes. If seminal fluid and milk slow the diffusion of solutes and salivary water, then mixing should enhance the diffusion. To test that possibility, we compared layers or mixtures of saliva and samples, at a ratio of 9 : 1 (0.9 mL of saliva plus 0.1 mL of milk containing $2 \times 10^6$ CEM lymphocytes). In the representative experiment shown in figure 4, mixing, compared with layering of saliva with milk, increased inhibition of virus yields. The findings indicate that mechanical mixing of milk with saliva may decrease inactivation of virus-infected leukocytes in milk, presumably by promoting diffusion of solutes and water.

Effect of saliva on cell-free HIV. For comparison with saliva’s inactivation of HIV-infected leukocytes, we studied the ability of saliva to inactivate any cell-free HIV that might occur in saliva. Previous reports showed a 3- to 5-fold inhibition of cell-free HIV by saliva [11–21], compared with the 10,000-fold inhibition of infected cells [9]. In our experiments, incubation of saliva at 37°C with cell-free HIV for 2 h resulted in an average 3-fold inhibition. This finding is in agreement with the previous reports and with the only moderate inactivation of free HIV.
by hypotonic fluids such as water [55–57]. Thus, saliva appears to inactivate cell-free HIV less strongly than it does HIV-infected leukocytes.

Effect of saliva on viability of macrophages. Our studies have used the human CEM lymphocyte cell line or normal PBMC that are mainly lymphocytes [9]. However, HIV also may be transmitted by infected macrophages [10]. Therefore, we determined whether normal human macrophages could be killed by saliva. Ficoll-hypaque–purified human mononuclear leukocytes were allowed to adhere to a glass surface for 3 h and then were treated with undiluted saliva or control medium for 15 min. The cultures were washed and stained with Wright’s stain and were observed for morphologic disruption [9]. The macrophages that were treated with saliva were >90% disrupted, as was found previously for lymphocytes and, more recently, also for macrophages [9, 10]. This finding indicates that macrophages as well as lymphocytes are inactivated by saliva.

Discussion

The rarity of salivary transmission of HIV has been reported to be due mainly to salivary inactivation of the HIV-transmitting leukocytes by the strong hypotonicity of saliva [9]. Saliva can inhibit leukocyte production of HIV by 10,000-fold in vitro, compared with the 3- to 5-fold reported inhibition of cell-free HIV for salivary inhibitors [9, 11–21]. This conclusion on cellular transmission is supported by the following evidence: most of the infectivity of HIV in the body fluids and secretions of carriers is found in infected leukocytes rather than as free infectious virus [58–62]; infected leukocytes may directly infect epithelial cells on mucosal surfaces [63–69]; any cell-free infectious HIV on mucosal surfaces is only weakly infectious for epithelial cells that lack the CD4 HIV receptor [64, 70, 71]; and simian mucosas, which lack the CD4 receptor in vivo, are 1000 times more resistant to cell-free simian immunodeficiency virus than is the simian bloodstream [71–73], which contains many CD4-positive leukocytes. Taken together, these observations provide strong evidence that the rarity of salivary transmission of HIV may be due mainly to saliva’s hypotonic inactivation of HIV-infected leukocytes in the mouth. In comparison with saliva, other mucosal secretions (e.g., vaginal) are isotonic; they maintain viability of HIV-infected leukocytes and are present at sites of transmission [9].

Previously unexplained is that oral transmission of HIV does occur epidemiologically if infected seminal fluid [29–34] or milk [35–39] is deposited orally, despite the presence of saliva. We hypothesized that orally deposited isotonic seminal fluid and milk could protect their contained, HIV-infected leukocytes by reconstituting the tonicity of saliva or by physically excluding the hypotonic saliva.

We found that reconstitution of tonicity by addition of seminal fluid, milk, colostrum, or balanced salt solution prevented salivary inactivation of virus-infected mononuclear leukocytes. Also, the physiologic volumes of seminal fluid, milk, and colostrum deposited in the mouth are sufficient to protect leukocytes against saliva and allow transmission of HIV. These findings are consistent with the hypothesis that the epidemiologic transmission of HIV orally by seminal fluid, milk, and colostrum may be due to their isotonicity, which prevents hypotonic salivary inactivation of the transmitting leukocytes.

Reconstitution of tonicity may be regulated by additional mechanisms. Because the mucosal secretions are more viscous than salt solution, they may physically sequester their leukocytes and thereby delay the salivary inactivation by impeding diffusion of hypotonicity. A sequestration mechanism of protection would predict that protection of leukocytes by seminal fluid and milk would be overcome by a longer incubation time, smaller droplets, and mixing. We found these predictions to occur, indicating that the salivary mechanisms of protection against HIV-infected leukocytes in orally deposited seminal fluid, milk, and colostrum are due to both reconstitution of tonicity and sequestration of infected leukocytes.

Thus, the oral cavity is unique among mucosal surfaces in having a defensive oral barrier to transmission of HIV and also probably to the human T cell leukemia virus [64–67, 74]. This main defense by salivary inactivation of the transmitting leukocytes, with the additional help of other salivary inhibitors [11–21], appears to account for the rarity of oral HIV transmission. The oral barrier can be overcome, however, by seminal fluid, milk, and colostrum, thereby accounting for the observed epidemiologic transmission by these mucosal secretions [29–39].

Also important medically is that the salivary defense has directed attention to the more probable mucosal transmitting agent—the HIV-infected leukocyte—rather than the less probable cell-free HIV. If the correct target to prevent mucosal trans-
mission is the HIV-infected leukocyte, then anticalcellular solutions that are even more potent than hypotonicity could be applied topically at other mucosal sites (e.g., vagina and rectum). At these other sites, the rate of vaginal transmission of HIV by seminal fluid is ~30% per year of exposure, whereas the risk of milk transmission of HIV to the newborn is ~15% [35–39]. Currently, we are studying a number of topical anticalcellular substances other than the reportedly ineffective nonoxynol-9 [72–74]. These candidate preventives include disinfectants for blood products, bile detergents, and commercial vaginal products, which are either naturally produced or used medically.

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References

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