A Model System of Oral HIV Exposure, Using Human Palatine Tonsil, Reveals Extensive Binding of HIV Infectivity, with Limited Progression to Primary Infection

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Oral exposure to human immunodeficiency virus (HIV) type 1 results in systemic infection, but many details surrounding virus transmission remain unresolved. We developed a mucosal model, using human palatine tonsil with intact external epithelium, to study events after oral exposure to HIV. When applied to the external epithelium, semen from an HIV-seropositive patient and cell-free virus both established HIV infection in individual tonsillar cells. However, clusters of infected tonsillar cells were detected where the epithelial surface was damaged. Investigation of the initial events in HIV transmission revealed extensive and stable binding of HIV virions and seminal cells to tonsil epithelium. In experiments modeling physiologically relevant events, the addition of seminal plasma resulted in enhanced virion binding to epithelial cells. These results indicate that, although extensive binding of HIV virions and seminal cells can be demonstrated at an exposed mucosal surface, there is only limited progression from binding to primary infection.

Oral transmission of HIV-1 is known to occur in 2 markedly different situations: receptive oral intercourse [1–4] and nursing by infants [5–7]. HIV infectivity is present in seminal fluid and milk from HIV-seropositive donors either as free infectious virions or as cell-associated infectivity [8–11]. To establish a new infection, HIV must cross the epithelial barrier of the oropharynx and/or gastrointestinal tract of the recipient, infect resident leukocytes, and then spread systemically.

The tonsil has been identified as a plausible portal for HIV entry after oral exposure, and virus-infected cells have been detected within the tonsil in both HIV-infected patients and simian immunodeficiency virus (SIV)–infected macaques [12–15]. The tonsil has also been implicated as the source of oropharyngeal shedding in HIV-infected individuals [16]. When the surface of macaque palatine tonsil was coated atraumatically with cell-free SIV, SIV-infected cells were first detected within the reticulated epithelium lining the tonsillar crypts [14]. These results correlate with the role of palatine tonsil as a secondary lymphoid tissue in which the tonsillar crypts are involved with sampling antigens introduced into the oral cavity [17]. Additionally, tonsillar leukocytes are susceptible to HIV infection when they are directly exposed to cell-free HIV [18, 19] (D.M., Z.-Q. Zhang, T.S., and P.S., unpublished data). Although these experiments illustrate the susceptibility of tonsillar leukocytes to HIV and SIV, many mechanistic details surrounding HIV transmission through biologically relevant routes have been difficult to study and remain unresolved.

Because oral HIV transmission occurs within a complex mucosal environment, we set out to study the initial events of oral HIV transmission by creating a mucosal model based on human palatine tonsil tis-
were mounted in agarose medium: a 1:1 mix of 4% agarose
HIV virions (virus particles/mL) containing green fluorescent protein (GFP) were generated by transfection by use of standard fluorescein filter sets.

MATERIALS AND METHODS

Virus stocks. A low-passage virus stock of cell-free HIV was prepared by in vitro passage of a primary patient isolate in heterologous peripheral blood mononuclear cells. This virus preparation (96–480) has a p24 content of 200 pg/mL and has dual tropic properties (P.S., unpublished data). Noninfectious HIV virions ($5 \times 10^{10}$ virus particles/mL) containing green fluorescent protein (GFP) were generated by transfection by use of PM-3 (a protease mutant) proviral DNA and vpr-GFP expression plasmids [20] (X.W., unpublished data). HIV-GFP virions were visible as discrete fluorescent particles at ∼600 magnification by use of standard fluorescein filter sets.

Tissue samples. Tissue samples obtained from routine surgical procedures performed at the Fairview University Medical Center (FUMC), Minneapolis, MN, were made available for research purposes with the assistance of the FUMC Tissue Procurement facility. Tissue samples were received within 1–3 h of the completion of the surgery, and processing in the laboratory was initiated promptly thereafter. Tonsil tissue with any visible macroscopic abnormalities was excluded. Routine histological analysis of the tonsil tissue revealed mild-to-moderate levels of hyperplasia. Informed consent was obtained from subjects who participated in the study. The human experimentation guidelines of the University of Minnesota were followed during the performance of the research.

Tissue pieces with external epithelial surfaces (∼25 mm²) were mounted in agarose medium: a 1:1 mix of 4% agarose and complete RPMI 1640 medium that contained 10% heat-inactivated fetal calf serum plus antibiotics—200 U/mL penicillin (base), 200 U/mL streptomycin (base), and 500 ng/mL amphotericin B (Antibiotic-Antimycotic 15240-062; Invitrogen)—such that the intact epithelial surface remained exposed and all cut surfaces were covered with agarose and, therefore, were protected from any contact with virions or cells that were applied to the external epithelium. The integrity of the agarose seal was confirmed by the following observations: (1) the fluorescent dyes Ethidium-Homodimer-II and Cell Tracker Orange (Molecular Probes) stained the exposed epithelium but did not stain the cut edges of the tissue pieces; and (2) analysis of the tissue focused primarily on the central surface of the tissue pieces, farthest away from the cut edges. However, when edges were analyzed, infected cells were not found adjacent to the lateral, cut edges. Under standard organ–culture conditions, the stratified epithelium remained in place for ∼24–48 h, and then sloughing was observed microscopically. Despite the sloughing of squamous epithelial cells from the luminal surface, the basal epithelial cells remained intact for 7–14 days, and epithelial cell proliferation was readily observed in response to deliberate disruption of the basal layer. The viability of tonsillar lymphocytes was assessed by physical disruption of the tonsil pieces that had been maintained in organ culture for various intervals, followed by trypan blue staining of the released single-cell suspension. There was a progressive decline in cell viability during the organ-culture incubations, and ∼50% of the lymphocytes remaining within the tissue pieces were viable after 5–7 days.

Tissue pieces in agarose were infected by careful application of the desired inoculum, such that only the drops of fluid came into contact with the tissue surface. The tissue surfaces remained moist throughout the infection period, but, in most cases, no deliberate effort was made to wash away the virus inoculum. Infections were terminated by flooding the agarose-embedded tissue pieces with 10% formalin. For some experiments, the epithelium was stained by use of 3 μg/mL of Ethidium-Homodimer-II, and laser scanning confocal images were collected from each tissue piece while it was still embedded in agarose. Fixed tissues were embedded in paraffin and sectioned (4 μm) for the general assessment of tissue architecture (by use of hematoxylin and eosin staining). Single-cell immunocytochemistry and in situ hybridization analyses were performed exactly as reported elsewhere [11, 21]. Cells were counterstained lightly with hematoxylin and mounted in Permount (Fisher Scientific) for microscopic evaluation.

Primary populations of epithelial cells were generated by randomly cutting tonsil tissue into small pieces (∼1–3 mm cubes) with either scissors and forceps or with a pair of scalpel blades. The resultant tissue pieces were cultured on Falcon cell-culture inserts (Becton Dickinson Labware) at the gaseous—
Figure 1. Infection of tonsil tissue through external epithelium with seminal cells from an HIV-infected donor (A and B) or cell-free HIV virions (C–E) 7 days after infection. Panels show immunohistochemistry to detect p24 antigen (brown) or in situ hybridization to detect HIV cytoplasmic RNA (E, black silver grains) (showing 1 representative of 4 independent tonsil infections with cell-free virus). Arrows, representative cells expressing p24 or HIV RNA.

Processing of semen samples. Freshly expressed semen samples were transported to the laboratory at room temperature, and processing was begun within 1–4 h of sample collection. Seminal cells were collected by centrifugation (250 g for 10 min at 15°C). The cell-free supernatant (seminal plasma fraction) was carefully removed and dispensed into Eppendorf tubes for long-term storage at −80°C. The seminal cell pellets were washed twice with Hanks’ balanced salt solution (30 mL/wash) and then resuspended in either (1) RPMI 1640 medium, 10% heat-inactivated fetal calf serum, and antibiotics (200 U/mL penicillin [base], 200 U/mL streptomycin [base], and 500 ng/mL amphotericin B [Antibiotic-Antimycotic 15240-062; Invitrogen]) for experimental infection transfer or (2) heat-inactivated fetal calf serum plus 10% dimethylsulfoxide for freezing. The infection experiments described here were initiated with nonfrozen material. For some experiments, viable seminal cells were labeled with 5-(and 6)-carboxyfluorescein diacetate succinimidyld ester (CFSE; Molecular Probes) by use of standard cell-labeling protocols. The population of seminal cells primarily includes spermatozoa and round cells that are composed of a mixed population of immature germ cells and leukocytes [22].

Immunofluorescence. The following antibodies were used at a 1:100 dilution: ZO-1, tight junctions (Zymed); AEI/AE3, pan cytokeratin (Chemicon); and clone Kal-1, HIV p24 (DAKO). Secondary antibodies were conjugated to cyanine 3 or 5 (Jackson ImmunoResearch Laboratories). Standard immunofluorescence procedures were used; briefly, primary populations of tonsil epithelial cells were fixed overnight in 10% formalin, washed, permeabilized with 0.3% Triton X-100, blocked with 1.5% donkey serum, and incubated with the appropriate primary antibody.
Seminal cells bind to external tonsillar epithelium. A and B, Representative laser-scanning confocal images from 3 independent experiments. The tonsil epithelium appears red (Ethidium Homodimer-II), and the seminal cells appear yellow (5- and 6-carboxyfluorescein diacetate succinimidyl ester). A, Brightest-point projection of 25 images captured at 15-μm intervals on the Z-axis, 5-day exposure to seminal cells. B, Brightest-point projection of 44 images captured at 2-μm intervals on the Z-axis, 3-h exposure to seminal cells. Panels A and B represent 2 separate combinations of tonsil and seminal cells from different donors. C–F, Five-micron cross-sections cut from the tissue shown in panel A. Nuclei, blue; seminal cells, green or yellow. C, Montage of low-power images showing the external surface (top) and the internal base of a series of crypts (pink or red fluorescence reveals cytokeratins and identifies cryptal epithelium). D, Low-power image showing seminal cells at the external surface of the epithelium. E and F, High-power images showing seminal cells that were found within the base of the crypt.

overnight at 4°C. Samples were then washed, reblocked, incubated with the appropriate secondary antibody for 1 h at room temperature, washed, mounted in Vecta-shield Hardset (Vector Laboratories) mounting medium, and stored at 4°C until viewed.

**Image collection and quantitative analysis.** Images were collected with a BioRad 1024 laser-scanning confocal microscope equipped with a krypton/argon laser and processed with Confocal Assistant (written by Todd Clerke Brelje, University of Minnesota, Minneapolis) and Adobe Photoshop software. For quantitative analysis, we randomly selected 5–10 fields of view, collected a Z series, and counted the number of particles per field according to threshold analysis and particle counting with Image J software (available at: http://rsb.info.nih.gov/ij/). A low but consistent level of background fluorescence (random fluorescent spots) is shown as the “average number of binding events” in samples that were not exposed to HIV-GFP. Student’s t test was performed to determine statistical significance.

**RESULTS**

To reconstruct the biological complexity associated with natural routes of HIV transmission, we developed organ-culture conditions that allowed the direct inoculation of HIV infectivity onto mucosal surfaces. Somewhat similar models have been used to study infection of the female reproductive tract [23–25]. The deliberate retention of intact epithelial surfaces and orientation-specific infection create an experimental context in which it is possible to document the transfer of HIV infectivity across a mucosal surface and to track the appearance of primary infected cells within exposed tissues.

We embedded freshly excised tonsil tissue in agarose medium, to cover all cut tissue surfaces and leave the external tonsil surface exposed. When we applied seminal cells from an HIV-seropositive donor to external tonsil epithelium, we detected occasional p24-positive cells within the recipient tissue in parallel but independent infections after 4 and 7 days (figure 1A and 1B). Three other combinations of fresh donor seminal cells and recipient tonsil tissue did not show detectable evidence of the transmission of HIV infectivity, reproducing the inherent variability that has been observed repeatedly in community-acquired HIV infections. The application of cell-free virus to external epithelium also resulted in individual p24-positive tonsillar cells; however, clusters of p24-positive cells were detected in areas where the tonsil epithelium appeared to be damaged (figure 1C–1E), as revealed by the absence of stratified squa-
Figure 3. HIV virions bind to external tonsillar epithelium. A–C, Representative laser-scanning confocal images. The tonsil epithelium appears red (Ethidium-Homodimer-II), and HIV–green fluorescent protein (GFP) virions appear green. A, External epithelium, intentionally scored with a scalpel blade to simulate a disrupted epithelium and exposed to HIV-GFP for 6 h. This panel is representative of 2 independent experiments. The image shows a brightest-point projection of 25 images captured at 10-μm intervals on the Z-axis. B, High-power image at the edge of the scalpel-blade scoring. The image shows a brightest-point projection of 69 images captured at 2-μm intervals on the Z-axis. C, High-power image of an undamaged area. The image shows a brightest-point projection of 47 images captured at 2-μm intervals on the Z-axis and is representative of 4 independent experiments.

HIV virions bind to external tonsillar epithelium. We performed additional infections with cell-free virus, to compare intact epithelium with intentionally damaged epithelium (scored with a scalpel blade) and observed similar results (2 independent experiments; data not shown). These results correlate with experiments performed with randomly cut pieces of tonsil, where robust infection occurred within tonsil pieces that lacked an epithelial surface [18, 19] (D.M., Z.-Q. Zhang, T.S., and P.S., unpublished data). The time interval required to establish HIV infection and the low numbers of infected cells detected when the epithelium remained intact both correlate with SIV-macaque mucosal infections [14, 26].

To document the initiating events of HIV transmission, we began to investigate the ability of HIV virions and seminal cells to bind to tonsil epithelium. We established conditions to label seminal cells with the live cell dye CFSE. When tonsil tissue, embedded as described above, was exposed to viable CFSE-labeled seminal cells in seminal fluid (both components derived from the same sample, donated by an HIV-seropositive individual) for intervals of 3 h–5 days, we observed many spermatozoa and round cells stably attached to the stratified epithelial cells (figure 2A and 2B). For the present analysis, we identified cell borders and nuclei by staining the tissue surface with Ethidium-Homodimer-II and then conducted laser-scanning confocal microscopy using a water-immersion lens while the tissue remained undisturbed in agarose. For further analysis, we systematically analyzed 8-μm cross-sections cut from the central areas of this tissue and found that both spermatozoa and seminal round cells had penetrated into deep areas of a tonsillar crypt (figure 2C–2F). Seminal cells that penetrate into these locations would be in close proximity to susceptible lymphocytes.

We used noninfectious HIV virions containing GFP to detect stable interactions between mucosal epithelium and the virions (figure 3). The accumulation of virions increased at sites that had been deliberately damaged (figure 3A and 3B), but binding could be readily observed in undamaged areas (figure 3C). Virion binding to tonsil epithelium also occurred in the presence of seminal plasma (data not shown). These results indicate that both the cell-free and cell-associated forms of HIV infectivity bind to the external epithelial surface and gain access to the tonsillar crypts.

As an essential step toward complete understanding of the process of HIV virion binding to epithelial cells, we developed a quantitative HIV virion–binding assay. To achieve greater consistency between experimental conditions, we used primary
tonsillar epithelial cells rather than tonsil tissue. Primary epithelial-cell populations are derived from small tonsil explants, and the outgrowing cells routinely express cytokeratins and form tight junctions (figure 4A). HIV-GFP virions bound to primary epithelial cells when they were exposed for 3 or 6 h and, relative to levels of binding in complete RPMI 1640 medium, HIV-GFP virion binding was enhanced in the presence of seminal fluid (figures 4B and 5A). Low levels of autofluorescence were sporadically detected in populations of primary cells that had not been exposed to HIV-GFP (figure 5A, experiments 2 and 3). However, the numbers of independent fluorescent particles were consistently higher for cells that had been exposed to HIV-GFP, which indicates that the majority of the signal could be attributed to the retention of HIV-GFP virions.

We also conducted additional binding experiments, with the goal of re-creating a natural flushing process that might remove bound virions from the epithelial surface of the tonsil. In these experiments, the epithelial cells that had been incubated with HIV-GFP virions were washed extensively and cultured for an additional 3–5 h. Approximately 50% of the virions that had bound at the outset remained bound to epithelial cells in this extended incubation protocol (figure 5B), which indicated that a substantial portion of the HIV-GFP virions that had initially bound remained tightly associated with the epithelial cells.

**DISCUSSION**

We have begun to exploit the utility of human organ–culture systems to dissect the cellular and molecular basis of the transmission of HIV infectivity. We have shown that HIV infectivity in seminal cells can traverse an external epithelial surface and initiate the infection of tonsillar cells in this ex vivo organ-culture system, in a direct reconstruction of exposure during receptive oral intercourse. To focus on the initial events that may lead to HIV transmission, we designed experiments to examine interactions between a natural HIV inoculum and tonsillar tissue. Seminal cells were found to bind to external epithelial cells and to penetrate into deep cryptal areas. We viewed spermatozoa and round cells to be surrogate markers for the potential distribution of cell-associated and cell-free sources of HIV infectivity on mucosal surfaces. Additionally, we have shown that HIV virions are able to bind to the external epithelial cells of tonsil tissue and can remain bound through several hours of additional culture. Seminal plasma did not inhibit this binding; instead, seminal plasma may actually have increased virion binding to epithelial cells. Bouhlal et al. [27] reported increased infection of the HT-29 colonic cell line in the presence of seminal plasma, in a complement-dependent manner. It is unclear whether the infection of epithelial cells plays a role in the initiation of a primary infection; however, it appears that the interactions between sources of HIV infectivity and the epithelium are strongly influenced by seminal fluid, and the effects of seminal fluid should be further studied.

During prevalidation experiments to establish quantitative HIV virion–binding assays, we observed that fluorescently labeled 0.1-μm-diameter latex spheres would also bind stably to mucosal epithelial surfaces and primary populations of epithelial cells. Some of the binding observed for HIV virions may,
Figure 5. Quantitative analysis of HIV virion binding to primary epithelial cells. A, Quantitative analysis of virion binding at 6 h (experiment 1, representative of 3 independent experiments with different tonsil and seminal plasma donors) and 3 h (experiments 2 and 3). B, Stable retention of bound HIV virions after washing and continuation in culture. In this experiment, matched primary epithelial-cell populations were incubated with HIV–green fluorescent protein (GFP) in undiluted seminal plasma, washed at 1 or 3 h, and either fixed immediately or continued in culture for an additional 3 or 5 h, before being washed, fixed, and analyzed for virion binding (representative of 3 independent experiments). Error bars show ±SE (n = 5-10). *P < .05, Student’s t test against the control sample. **P < .05, Student’s t test, HIV-GFP plus medium vs. HIV-GFP plus seminal plasma. ND, not determined.

Therefore, reflect the trapping of virus particles or nonspecific mechanisms that cause particulate matter to bind to epithelial cells. However, both specific and nonspecific mechanisms that lead to the stable retention of virion particles at mucosal surfaces may ultimately contribute to the transmission of HIV infection. We are currently using blocking antibodies to identify key components in the virion envelope and at the epithelial cell surface that contribute to stable virion binding.

Although reports have described HIV infection [28–30] and HIV transcytosis [31, 32] by cultured monolayers of immortalized epithelial cells, to our knowledge, this is the first study to have explored the ability of HIV virions and seminal cells to bind to tonsil epithelium. Our results establish a distinct separation between extensive and rapid binding events and the relatively rare establishment of primary infection in recipient tissue, and they highlight the protective capabilities of an intact mucosal epithelium. The results of epidemiological studies have indicated that ~1 case/200–1000 exposure events leads to primary infection for male-to-female heterosexual HIV transmission [33], and the probability of transmission resulting from oral exposure may be even lower [34]. Thus, the binding of HIV infectivity at mucosal surfaces does not automatically lead to primary infection, but such binding can be regarded as an essential prerequisite for the successful transmission of HIV. Sources of HIV infectivity associated with epithelial cells may remain infectious [35, 36], thereby increasing the time frame...
for establishing primary infection in the recipient. Additionally, we believe that the surface binding of seminal cells and HIV virions may induce transient perturbations in epithelial integrity that facilitate the transmission of HIV and that such mechanisms can only be recognized in the context of biologically relevant sources of HIV infectivity.

By virtue of requiring surgical removal, tonsil tissues cannot strictly be classified as “normal,” but, given the frequency of chronic tonsillitis in children and young adults and sleep apnea in middle-aged men, it is not inconceivable that tonsillar inflammation and enlargement may contribute to the overall process of oral HIV transmission. Ongoing immune responses within the exposed tonsil may affect susceptibility to de novo HIV infection by >1 mechanism. The activation of resident leukocytes and/or recruitment of activated leukocytes from distal sites could facilitate the initial establishment of HIV infection. However, more subtly, leukocyte activation in an inflammatory response may cause rapid and direct changes in the structure of the external surface of the tonsil and thereby increase the probability that exposure to HIV infectivity will actually progress to primary HIV infection. The induced proliferation of resident tonsillar leukocytes and the recruitment and proliferation of other leukocytes will result in tonsillar enlargement and may perturb the tonsillar surface in such a way that the reticulated epithelium, which was previously confined to the surface of cryptal fissures, then makes up part of the external, exposed surface of the tissue [17]. As a consequence of this type of tissue reorganization, the reticulated epithelium would be much more accessible or vulnerable to HIV infectivity, and the probability of initiating a primary HIV infection could be increased significantly. By analogous reasoning, preexisting inflammatory conditions, induced by sexually transmitted diseases in the female reproductive tract or rectal tissue, could result in alterations to the cellular composition of the surface epithelium and have a direct impact on susceptibility to primary HIV infection [37].

The infection of human tissue in organ culture does not exactly match the initiation of infection in a newly exposed patient, because there is no communication between the exposed piece of tissue and the rest of the body. However, the absence of an induced inflammatory response that involves cell recruitment from distal sites allows the sequence of primary events to be studied, in isolation, at the site of exposure. Although we have focused on palatine tonsil, significant correlates can be identified with other oral epithelial surfaces, including the other components of the Waldeyer’s ring of lymphoid tissue [17], that could come in direct contact with HIV infectivity during oral HIV exposure. Additionally, because of structural similarities between epithelial surfaces in different tissues, the present results in the tonsil may also be predictive of events that occur at other mucosal sites exposed to HIV infectivity [38]. A more complete understanding of the events immediately following exposure will be of paramount importance in evaluating vaccination and other interventional therapeutic strategies that may confer protection at mucosal surfaces.

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References