Coming of age: reconstruction of heterosexual HIV-1 transmission in human *ex vivo* organ culture systems

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Heterosexual transmission of human immunodeficiency virus-1 (HIV-1), from men to women, involves exposure to infectious HIV-1 in semen. Therefore, the cellular and molecular processes that underlie HIV-1 transmission are closely interconnected with fundamental principles of human reproductive biology. Human *ex vivo* organ culture systems allow experimental reconstruction of HIV-1 transmission, using human semen and premenopausal cervicovaginal mucosal tissue, with specific emphasis on the progression from exposure to development of primary HIV-1 infection. Clearly, an isolated piece of human tissue cannot duplicate the full complexity of events in natural infections, but with correct observation of conventional medical and ethical standards, there is no opportunity to study HIV-1 exposure and primary infection in young women. Human mucosal organ cultures allow direct study of HIV-1 infection in a reproducible format while retaining major elements of complexity and variability that typify community-based HIV-1 transmission. Experimental manipulation of human mucosal tissue both allows and requires acquisition of new insights into basic processes of human mucosal immunology. Expanding from the current foundations, we believe that human organ cultures will become increasingly prominent in experimental studies of HIV-1 transmission and continuing efforts to prevent HIV-1 infection at human mucosal surfaces.

INTRODUCTION

The molecular virology underlying human immunodeficiency virus-1 (HIV-1) infection has been elegantly described over many years, and a fundamental understanding of the HIV-1 virus replication cycle in infected CD4+ T cells[1] has been critical in the development of inhibitory drugs that are used for current antiretroviral therapy (ART).[2] Important ongoing molecular studies are continuing to reveal complexities in the HIV-1 infectious process—such as the interplay between HIV-1-encoded accessory proteins and host-encoded proteins,[3–5] with implications for the development of new classes of anti-HIV-1 agents. However, progress has been more limited in the acquisition of fundamental understanding of the biological events surrounding HIV-1 transmission and primary infection that could be used to guide the development of vaccines and/or microbicides to protect against mucosal exposure and initiation of HIV-1 infection.[6] Basic studies on virus transmission have been conducted in primate hosts, principally in rhesus macaques (*Macaca mulatta*), using simian immunodeficiency virus (SIV) and a vaginal route of infection of female animals, which resembles heterosexual HIV-1 transmission into young women.[7,8] The SIV/macaque model has been especially useful for studies of virus amplification and dissemination from sites of initial infection, virus replication in lymphoid tissue, and disease progression as CD4+ T-cell numbers decline. However, there are many important biological differences between human HIV-1 heterosexual transmission and experimental SIV infection of female rhesus macaques, relating to exposure to semen and frequency of unprotected intercourse, sexually transmitted infections, and extended use of hormonal contraceptives, which affect mucosal barrier function and overall cellularity in the female reproductive tract (FRT).[9–13] Although these human variables cannot be easily controlled, variability from person to person must be recognized as a major component influencing primary HIV-1 infection in young women, so that comprehensive protective strategies can be designed

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with a complete awareness of the biological reality of the human condition.

On one level of understanding, the mechanism of heterosexual HIV-1 transmission from an HIV-1-infected male donor to an uninfected female recipient is well defined: infectious HIV-1 shed in semen is deposited onto mucosal surfaces in the FRT and primary HIV-1 infection becomes established in mucosa-associated leukocytes.[14–16] However, there are multiple variables relating to both the male infected donor and the female recipient that profoundly affect the transmission process. It has been estimated that male-to-female HIV-1 transmission occurs for 1 in 200 to 1 in 1,000 unprotected exposure events.[17,18] However, it is clear that a single exposure can be sufficient to transfer infection and that high viral loads in semen have been associated with increased risk of transmission.[19] There is considerable uncertainty regarding the sources of infectious HIV-1 in the male reproductive tissue: some HIV-1-infected men shed infectious HIV-1 virions in semen, whereas other infected men shed HIV-1-infected cells in semen.[20–25] Depending on ART status, the highest levels of HIV-1 infectivity (cell-free infectious virions or cell-associated infectivity in the form of infected cells) are detected in semen in the interval after seroconversion. As blood viral loads decrease in response to adaptive anti-HIV-1 immune responses, so do semen viral loads. Early intervention with ART would have an immediate impact in decreasing blood viral loads, and a somewhat slower impact in decreasing semen viral loads.[26] In the exposed female recipient, multiple variables relating to the integrity of epithelial surfaces have been recognized, which affect susceptibility to primary HIV-1 infection.[9] In the FRT, pre-existing physical damage caused by trauma during intercourse[27–29] or by independent ongoing viral infections (herpes simplex viruses types 1 or 2) can disrupt the integrity of the mucosal epithelium and allow direct contact between sources of HIV-1 infectivity and leukocytes in the submucosa.[30] Independently, or in addition, relatively common conditions such as bacterial vaginosis[31–33] or vaginal candidiasis[34,35] may contribute to mucosal inflammation and cause profound alterations in the vaginal microenvironment by displacing commensal *Lactobacillus* spp. and by increasing the basal pH from the standard acidic range, pH 4–5, toward neutrality.

Unprotected intercourse has also been recognized as contributing to FRT disturbance by triggering rapid recruitment and release of neutrophils across the endocervical epithelium, in a process that has been linked to the removal of excess spermatozoa. Neutrophil recruitment after unprotected intercourse is not easily studied in young women,[36] but has been investigated extensively in the context of animal artificial insemination programs.[37,38] Mild-to-moderate cervicitis is commonly observed in sexually active young women in whom inflammatory infiltrates may be quite localized or diffusely distributed in the submucosa.[39–41] The submucosal infiltrates consist of variable leukocyte populations, but activated CD4+ T cells are consistently detected in proximity to the luminal surface. Multiple mechanisms have been proposed to explain HIV-1 transfer across an apparently intact epithelial barrier: infection of activated Langerhans cells (LCs),[42,43] infection of macrophages,[44] or transcytosis across epithelial cells.[45] However, if the epithelial barrier has been damaged, then it is unnecessary to invoke any of these active mechanisms to explain how HIV-1 infectivity might come into direct contact with susceptible CD4+ T cells in the submucosa.

Table 1 ***Ex vivo*** **HIV-1 infection in human cervicovaginal organ cultures: short-term experiments focusing on HIV-1 transmission and primary infection**

Significant research benefits
Ostensibly normal human premenopausal tissue for direct experimentation
Reconstruction of natural routes of human exposure and primary infection
Design and evaluation of anti-microbial strategies with human target cells
Foundation to study microbial synergy in human polymicrobial infections
Insights relate to human condition and human susceptibility to infection
Concerns and limitations of current human organ culture systems
Tissue availability
Donor-to-donor variability
Retention of tissue architecture/cell viability for duration of organ culture

Abbreviation: HIV-1, human immunodeficiency virus-1.
No opportunity to study dissemination of HIV-1 infection and disease progression.
No opportunity to study immune cell recruitment from distal sites.
These issues are deliberately excluded from the current experimental format because of requirements for longitudinal access to patient materials and/or access to primate infection models.
The absence of immune cell recruitment does allow local events at the portal of entry to be studied without the impact or influence of cells from distal sites.

This review presents a general rationalization for the research opportunities provided by *ex vivo* experiments with human mucosal tissues (**Table 1**). The underlying premise is that acquisition of fundamental information relating to primary HIV-1 transmission can best be derived from direct study of the components of human tissue systems. Clearly, it is totally inconceivable to contemplate deliberate exposure and infection of human patients. Any patient seeking medical advice because of potential exposure to HIV-1 is likely to be offered ART immediately, in conjunction with future serological testing to determine whether seroconversion has actually occurred. Therefore, the opportunities to study HIV-1 exposure and primary infection in patients are severely limited by medical considerations, but the development of *ex vivo* human organ culture systems has created a valuable experimental platform for direct study of heterosexual transmission of HIV-1 in the human system.

BASIC EPITHELIAL STRUCTURES

The cellular composition and histological structure of epithelial surfaces in the adult human premenopausal FRT vary according to anatomical location, hormonal signals relating to

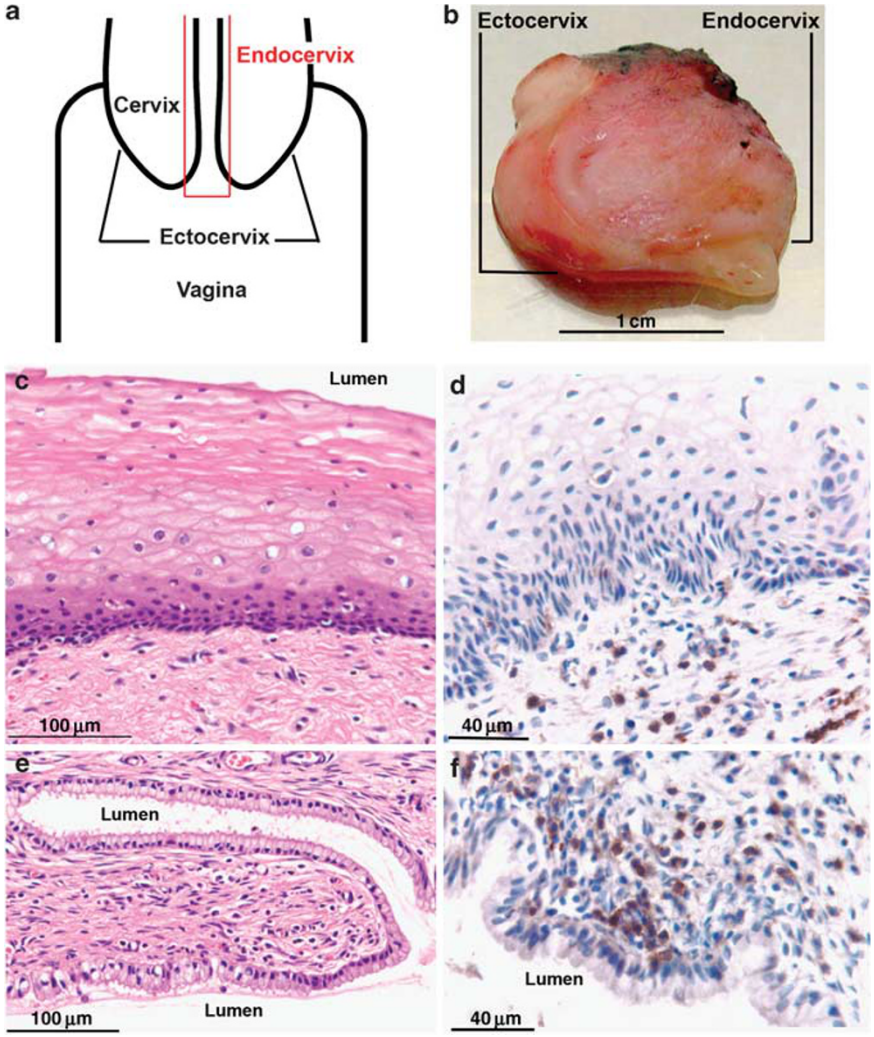

Figure 1 Representations of "normal" human premenopausal cervicovaginal tissue. (**a**) Line drawing of the head of the vaginal vault and the uterine cervix in a longitudinal section. (**b**) Typical wedge-shaped cervicovaginal tissue sample. (**c**) Hematoxylin and eosin (H&E) staining to show the multi-layered stratified squamous epithelium of the ectocervix. (**d**) Immunocytochemical (ICC) detection of CD4+ T cells (brown stain) situated just beneath the basal cell layer of the ectocervical epithelium. CD4+ T cells are the primary target cells for HIV-1 infection. Hematoxylin counterstain reveals all cell nuclei (blue). (**e**) H&E staining to show the single cell layer columnar epithelium of the endocervix. (**f**) ICC detection of CD4+ T cells (brown stain; cell nuclei counterstained blue with hematoxylin) situated immediately beneath the columnar epithelium of the endocervix. HIV-1, human immunodeficiency virus-1.

the menstrual cycle, and parity (child-bearing history).[15] The vaginal walls are composed of a multi-layered stratified squamous epithelium. The outermost cell layers contain dead and dying cells with a high glycogen content that are sloughed away and continuously replaced by outward displacement of terminally differentiated cells from below. Cell proliferation is confined to the suprabasal cell layer—cells situated immediately above the continuous basal cell layer.[46] Typically, the vaginal epithelium is 15–30 cell layers thick, and at mid-cycle, during the interval of maximum fertility, the luminal surface is usually coated with a surface layer of keratin. The multi-layered stratified squamous epithelium of the vaginal walls creates a continuous surface with the ectocervical epithelium, although there is generally less variability in the thickness of the ectocervical epithelium, and the surface layers of the ectocervical epithelium may not become keratinized. In the vicinity of the cervical os, the lower opening into the uterine cervix, the mucosal surface changes abruptly into a single cell layer columnar epithelium that defines the anatomical endocervix. The boundary between the stratified squamous epithelium of the ectocervix and the columnar epithelium of the endocervix is known as the cervical transformation zone. The positioning of the cervical transformation zone relative to the cervical os varies according to age, hormonal signals, and pregnancy. Around the time of menarche, 80–90% of adolescent girls exhibit cervical ectopy, a normal condition in which the columnar epithelium extends beyond the cervical os.[47,48] Over time, the external areas of the columnar epithelium are gradually replaced by the stratified squamous epithelium such that the columnar epithelium is typically confined to the endocervical canal in young adult women (**Figure 1**). Cervical ectopy also occurs during pregnancy with restoration of the stratified squamous epithelium after delivery.

Tissue surface remodeling is associated with active epithelial cell proliferation, with the practical consequence that the cervical os and transformation zone are routinely sampled using a cytological brush to collect cells for Papanicolaou smear tests and for visualization of aberrant cell morphologies. Dysplastic changes in the transformation zone are the first recognized signs of human papilloma virus infections that may progress to cervical carcinoma.[49,50]

In considering the protective capabilities of FRT mucosal barriers, the single columnar epithelial cell layer of the endocervix would appear to be more vulnerable to infection than the multi-layered epithelial surfaces of the vaginal walls and ectocervix. The transient condition of cervical ectopy, in which the columnar epithelium extends beyond the cervical os,[47,48] may account for the particular susceptibility to HIV-1 reported for adolescent young women who may be coerced into early sexual activity by cultural practices, including marriage early in life.[51–54] In addition, the common occurrence of mild-to-moderate cervicitis in sexually active young women results in the placement of inflammatory cells, including activated CD4+ T cells in close proximity to the mucosal surface with the consequence that HIV-1 virions and/or HIV-1-infected cells may only need to penetrate 1–5 cell layers to encounter susceptible target cells (most probably CD4+ CCR5+ T cells) beneath a columnar epithelial surface.[39,55] Infection may also occur across a stratified squamous epithelial surface[56–58] where the likelihood of infection would increase with epithelial thinning or with epithelial damage, as might arise from physical trauma or unrelated microbial infections. Small numbers of CD4+ T cells can be observed, either spread diffusely or localized to one particular site, within or immediately beneath intact stratified squamous epithelial structures, presumably reflecting previous or ongoing immune responses. More highly organized arrangements of leukocytes, tertiary lymphoid structures, are periodically detected in the submucosa of the vagina and ectocervix,[59] but the signals required to establish and potentially disaggregate these structures currently remain unclear. Collectively, recognition of extensive structural variability across and immediately beneath a mucosal tissue surface leads to the realization that susceptibility to HIV-1 infection may also be highly variable across this tissue surface.

Various studies, extending from vaginal infections of hysterectomized female macaques[60] to human *ex vivo* organ cultures discussed here, have demonstrated that SIV or HIV-1 infection can occur across the stratified squamous epithelium of the vaginal walls.[6] However, these studies do not establish the frequency with which community-acquired HIV-1 infection actually does occur across the vaginal epithelium. If the intact epithelium has been damaged, e.g., by trauma during intercourse,[27–29] special practices such as "dry sex,"[61,62] or by the presence of herpetic lesions,[63] then the risk of HIV-1 exposure progressing to primary infection is much increased. LCs and macrophages, both potential targets for HIV-1 infection and/or HIV-1 virion capture or internalization, are randomly distributed in small numbers throughout the stratified squamous epithelium of the vaginal walls and the ectocervix.[42–44,64] Dendritic processes from LCs extend into the luminal space to capture antigens, and then LC migration to the draining lymph node could trigger a priming reaction for naive T cells. This normal mechanism of antigen sampling is subverted by HIV-1 under conditions of LC activation as LCs become susceptible to HIV-1 infection and may facilitate establishment of primary infection by virus amplification and subsequent transmission to CD4+ T cells.[42,43] It is also possible that macrophages within stratified squamous epithelial membranes could support an initial round of HIV-1 infection immediately after exposure, but it remains unclear how localized infection in macrophages might be further amplified.

IMMUNE COMPONENTS AT EPITHELIAL SURFACES

Innate immunity

A wide variety of innate protective mechanisms have been described at epithelial surfaces,[65–68] but just as there are key structural differences in epithelial composition so there is corresponding variability in the subset of innate components that might function at any given epithelial surface (**Table 2**). Protective mechanisms can be divided between secreted components (such as mucus, complement, defensins, lactoferrin, lysozyme, secretory leukocyte protease inhibitor), cell-associated components (such as cell surface cilia and pattern-recognition receptors, including Toll-like receptors and C-type lectins, such as Langerin and DC-SIGN), and inducible secreted components (such as interleukin-6, interleukin-8, macrophage inflammatory protein-3α, and interferon-α). Different assays have been developed to show protective efficacy in the context of carefully defined *in vitro* systems. However, assays showing biochemical inhibition of HIV-1 infection in a cell culture system using defined medium and immortalized mammalian cells may have only limited connection to the biological reality of HIV-1 infectivity in semen that is coating mucosal surfaces in the human FRT. These concerns are well illustrated by the finding of a correlation between genital infections, leading to induction of α-defensins and LL-37 and an increased risk of HIV-1 infection.[69] It is also likely that there will be quantitative and qualitative differences in innate immune responses across different populations that, at least in part, will reflect individual behavior patterns within any susceptible group and past/ongoing FRT exposure to microbial agents. The overall uncertainty surrounding innate protective components is highlighted by the disturbing conclusion that innate components were seemingly insufficient to protect individuals exposed to HIV-1, which constitute the ~3,500 new infections occurring daily in young women.

In experimentally infected female macaques, an "outside-in" mechanism of innate signaling has been recognized whereby initial exposure to a high-titer cell-free SIV inoculum triggers induction of macrophage inflammatory protein-3α and the subsequent recruitment of plasmacytoid dendritic cells to mucosal surfaces.[70] Further signaling through plasmacytoid dendritic cell-derived interferon-α has been proposed as a mechanism that recruits new target cells into the submucosa with the consequence of amplifying local SIV replication. These results lead

Table 2 Mucosal cells involved with HIV-1 (SIV) exposure and primary infection

Host cell type	Host species	Experimental system	Primary role in transmission
CD4+ T cells	Human	Patient samples, PBMC	Major target cell supporting HIV-1 replication
Macrophages	Human	Patient samples, monocyte-derived macrophages from PBMC	Susceptible to HIV-1 infection Possible long-term reservoir of virus infection[44]
Dendritic cells (DCs)	Human	Primary cell populations, organ culture	Virus transfer to draining lymph nodes, cytokine/chemokine release[64,172]
Langerhans cells (LCs)	Human	Primary cell populations, organ culture	Virus uptake across stratified squamous epithelial surfaces Cytokine/chemokine release[42,43]
Epithelial cells	Human	Primary cell populations, immortalized cells	Transcytosis, possible transit reservoir, cytokine/chemokine release[45,65,188]
CD8+ T cells (CTLs)	Human	Patient samples	Protection—lysis of infected host cells[7,189]
Natural killer (NK) cells	Human	Patient samples, *ex vivo* stem cell derived (iPSCs and hESCs) populations	Protection—lysis of infected host cells[87,88,90]
Plasma cells	Human	Patient samples	Secretory IgA-mediated protection in exposed seronegative individuals[66]
γδ T Cells	Human, Macaque	Patient samples	Innate immune function in stratified squamous epithelium[43]
Regulatory T cells (Tregs)	Macaque	SIV-infected macaques	Downmodulation of anti-SIV CTL responses[190]
Stromal cells	Human	Primary cell populations	Possible target/reservoir for HIV-1 infection Cytokine/chemokine release[188]
Neutrophils	Human	Patient samples	Recruited by epithelial stress responses and semen[36,191]
Plasmacytoid dendritic cells (pDCs)	Human, Macaque	Patient samples, SIV-infected macaques	Recruited by epithelial stress responses Cytokine release[70]

Abbreviations: hESC, human embryonic stem cell; HIV-1, human immunodeficiency virus-1; iPSC, induced pluripotent stem cell; PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus.

to the counterintuitive conclusion that downmodulation of a local innate response may contribute to increased protection against mucosal challenge, and studies are ongoing to understand the immunomodulatory properties of glycerol monolaurate, the agent used in these macaque studies.[70] There is no clear indication of the extent to which "outside-in" innate signaling may apply to sexually active young women, given the common occurrence of cervicitis and the finding of numerous plasmacytoid dendritic cells in proximity to the mucosal surface and distributed throughout the submucosa of the endocervix (JE Horbul, SC Schmechel, BRL Miller, SA Rice, and PJ Southern, unpublished data). In addition, exposure to seminal plasma has been shown to induce macrophage inflammatory protein-3α (CCL20) secretion from epithelial cells,[71,72] suggesting that frequent unprotected intercourse would repeatedly signal for plasmacytoid dendritic cell recruitment to the endocervical surface.

Multiple studies have attempted to define correlates of induced immunity leading to protection from SIV challenge in immunized macaques[73–77] or that might account for protection in women who are known to have been exposed to HIV-1 but who show no evidence of seroconversion,[78] or in women who stringently control HIV-1 replication in the absence of ART.[79–81] It is reasonable to infer that innate components may have exerted protective effects, but this is difficult to quantify other than by comparison with groups of women at very low risk for repeated HIV-1 exposure. Furthermore, protective mechanisms that have been identified in the macaque system may not be equally applicable in the human FRT, as shown by a nonsense mutation in the coding sequence for human θ-defensin[82,83] and a single amino-acid change in human tripartite motif protein 5α that eliminates the anti-HIV-1-inhibitory activity demonstrated by simian tripartite motif protein 5α.[84–86]

The connections between natural killer (NK) cells and virus infection have been studied in many different systems, but there is now increasing interest to evaluate NK-mediated protection against HIV-1 infection.[87,88] Experimental NK studies have been propelled by recent findings that human NK-like cells can be derived from human embryonic stem cells.[89] NK cell populations from human embryonic stem cells or from induced pluripotent stem cells, may have therapeutic application in control of ongoing HIV-1 infection because *in vitro* studies have shown suppression of HIV-1 infection by three different mechanisms: direct lysis of HIV-1-infected cells, antibody-dependent cellular cytotoxicity, and through production of chemokines and cytokines.[90] It is also conceivable that induction and expansion of NK populations at exposed FRT mucosal surfaces could contribute directly to resistance to HIV-1 infection by elimination of infected cells before amplification and virus dissemination can occur.

Adaptive immunity

The distribution of cellular and soluble components from the adaptive immune system is likely to be highly variable at mucosal surfaces within the FRT, in part because of the variable composition and distribution of inflammatory cell infiltrates (**Figure 1**, **Table 2**). There is some evidence suggesting that regulatory T cells may modulate antigen-specific mucosal immune responses leading to an unpredictable outcome: restricted numbers of available target cells could delay or prevent the establishment of primary infection,[91] whereas restricted numbers of antigen-specific cells at the portal of entry could facilitate the establishment of primary infection.[92] At present, basic characterization of human, antigen-specific regulatory T cells and signals to induce or to expand regulatory T cell activity at mucosal surfaces remain in the very earliest stages of investigation.[93] The presence of any type of HIV-1-antigen-specific cells in proximity to mucosal surfaces in women who would conventionally be described as seronegative implies previous exposure to HIV-1, without the establishment of disseminated infection. Groups of women working in the sex trade in Nairobi have been identified, who remained seronegative despite repeated exposure to HIV-1.[78] The immunological basis for this sustained protection from infection seems to involve both anti-HIV-1-specific sIgA[94–97] and anti-HIV-1-CD8+ T cells (CTLs).[98] Tragically, a change in lifestyle for some members of this group of Kenyan women, resulting in much reduced probable exposure to infectious sources of HIV-1, subsequently led to the establishment of HIV-1 infection and seroconversion.[99] Other studies, based on different groups of exposed and non-infected women, have confirmed the importance of mucosal IgA,[100] failed to detect specific mucosal antibody responses,[101] or concluded that immunological protection against systemic infection was provided by mucosal HIV-1-specific IgG.[102] No immediate resolution has been proposed for the divergence in findings that has been attributed to either mucosal IgA or IgG.[66,103]

PROGRESSION FROM HIV-1 EXPOSURE TO PRIMARY HIV-1 INFECTION

In experimental FRT infections, there is a delay between virus exposure and detection of newly infected cells. This finding applies to *ex vivo* human organ cultures and vaginal infections of macaques—in both HIV-1 and SIV infections, an interval of 3–5 days is required for consistent detection of newly infected cells by *in situ* hybridization or immunocytochemistry.[92,104,105] However, a sequence of events ultimately leading to infection, resistance to infection, or failure to establish infection will probably be initiated as soon as the infectious inoculum comes into proximity to the mucosal surface. In the SIV-female macaque system, a vaginal douche has been used shortly after high-dose SIV exposure to inactivate SIV virions remaining within the lumen of the FRT or bound to the mucosal surface, and this treatment did not affect the initiation of SIV infection.[106] These findings indicate that events progressing to primary SIV infection and, by implication, primary HIV-1 infection begin shortly after mucosal exposure in the FRT, but this does not mean that the infectious process always begins immediately. However, recognition that critical events could begin shortly after exposure has established a requirement to consider physical events occurring at exposed mucosal surfaces. Initial events following virus exposure are more likely to be influenced by principles of biophysics—movement, trapping, and binding of particles in a complex fluid distributed across irregular surfaces—than by recognized mechanisms of molecular virology. We have used a multi-microscope approach to visualize a dynamic sequence of events that occurs when HIV-1 virions or HIV-1-infected cells are deposited onto intact human epithelial surfaces.[105,107] In many experiments, fluorescently labeled latex particles (0.1 and 4.0 μm diameter spheres; Invitrogen: Molecular Probes, Eugene, OR; approximate representations of HIV-1 virions and HIV-1-infected lymphocytes, respectively) have been used to demonstrate that inert particles readily interact with mucosal surfaces in the FRT. Surface binding and tissue penetration were examined by time-lapse confocal microscopy, using fresh unfixed human tissues that contained live cells. Subsequently, after tissue fixation and processing, thin sections cut with known orientation from the same tissue pieces were examined by conventional light or fluorescence microscopy, at high resolution. By image analysis, it was possible to enumerate virus particles and cells that remained tightly bound to epithelial surfaces after extensive washing, and this has been used for quantitative assessment of inhibition of HIV-1 virion binding.[105] In addition, it is now possible to visualize interactions between different classes of microbes at epithelial surfaces and to investigate the cellular and molecular basis for any increased susceptibility to infection (microbial synergy), when multiple microbes are present[108–110] (JE Horbul, SC Schmechel, BRL Miller, SA Rice, and PJ Southern, unpublished data).

VARIABILITY AND HETEROGENEITY AT MUCOSAL SURFACES

The mucosal surfaces of the premenopausal FRT are directly responsive to hormonal signals throughout the menstrual cycle. Around the time of ovulation in mid-cycle, the outermost layers of the vaginal epithelium can develop a surface layer of keratin. This cyclical change has been rationalized as a mechanism to protect the vaginal walls from trauma during intercourse, occurring during the short interval of maximum female fertility. Concurrently, mucus secretion from the endocervical epithelium changes such that mid-cycle mucus is highly hydrated (viscosity is at the lowest level),[111] presumably to facilitate movement of spermatozoa through the endocervical canal and into the fallopian tubes. There is a clear protective role for mucus in preventing direct contact with the epithelial cell surface, but particles suspended in mucus may remain in proximity to the epithelial cell surface for extended intervals,[105] increasing the likelihood that specific binding to the surface may then follow. Recent studies have recorded a 60–70-fold increase in the diffusion rate for HIV-1 virions in cervical mucus that is associated with an increase from approximately pH4 to approximately pH6, as would occur immediately after intermixing of mucus and semen.[112] These findings emphasize the dynamic nature of interactions between components of semen, including HIV-1

infectivity, and mucosal surfaces in the FRT and indicate that further studies involving semen and mucus are warranted.[113]

ANALYTICAL TOOLS: SINGLE CELL DETECTION TECHNOLOGIES

Heterogeneity in tissue microarchitecture that is typically present at mucosal surfaces has the potential to create technical challenges for experimental analyses in which events within one cell may be substantially affected by the surrounding cells. The importance of hierarchical interactions is best appreciated by single-cell analytical methods—*in situ* hybridization and immunocytochemistry—in which the anatomical context of infected cells can be readily visualized.[104] These procedures require considerable time and skill to retrieve high-quality information under optimized conditions. Multiple sections must be surveyed to develop a comprehensive reconstruction of exposure/infection at the mucosal surface. As an independent experimental approach, several different procedures have been developed for enzymatic digestion of mucosal tissues for subsequent analysis of single cell suspensions or nucleic acid and protein extractions.[114,115] However, all of these disruption and extraction procedures eliminate spatial information in which, at least during exposure, proximity to the mucosal surface will dramatically increase the likelihood of involvement in the initiation of infection. The power and efficiency of delineating functional cell populations by fluorescence activated cell sorting are undermined by the absence of information relating to location, and if the sorting parameters are set to exclude multi-cell complexes, any functional detail relating to cell–cell interactions may be overlooked.

Refinements to the well-established *in situ* hybridization and immunocytochemistry single cell analytical procedures include *in situ* tetramer hybridization[116] and laser capture microdissection (LCM).[117] *In situ* tetramer hybridization allows visualization of the juxtaposition of antigen-specific CTLs, detected by tetramer staining of CTLs recognizing immunodominant epitopes[73] and virus-infected cells, visualized by conventional *in situ* hybridization procedures. Application of *in situ* tetramer hybridization to mucosal and lymphatic tissue samples from acute SIV infections has revealed low *in vivo* effector-to-target ratios (< 2) for SIV-specific CTLs recognizing immunodominant epitopes, and provides an immunological explanation for the inability of primary SIV-specific CTL responses to contain the SIV infection.[7,116] LCM involves brief antibody staining of frozen or fixed tissue sections, followed by laser capture of microscopically selected areas that are then extracted to obtain minute amounts of nucleic acid for reverse transcription-PCR or PCR amplification or used for direct protein analysis.[117] Individual infected cells have been identified in HIV-1-infected brain sections by LCM analysis,[118] and it seems probable that new insights will emerge from LCM-based studies of exposed mucosal surfaces. Although *in situ* tetramer hybridization and LCM are technically demanding, these procedures provide valuable opportunities to derive molecular and cellular information relating to function, which can be superimposed onto a spatial framework.

A number of organ culture studies based on punch biopsies (typically 5 mm) or cut tissue blocks taken from the same surgical tissue sample have established biochemical outcomes for induction of changes in host cells or HIV-1 infection by analyzing cytokines, virions, and/or soluble viral proteins that are released into the culture medium.[115,119,120] Assays based on enzyme-linked immunosorbent assay or multiplex bead procedures to measure soluble antigens, or co-culture with susceptible target cells to demonstrate the presence of infectious HIV-1 yield quantitative information, but a key biological element is missing in terms of identifying the cell type and physical location of the source of target molecules. An additional complication arises from cell loss or active cell emigration from cut tissue surfaces, so that accumulation of soluble components in the culture medium during organ culture incubations needs to be interpreted with caution. More importantly, loss of structural integrity during *ex vivo* incubations may affect the overall experimental outcome and diminish the connection to an exact reconstruction of HIV-1 transmission at human mucosal surfaces.

PRACTICAL CONSIDERATIONS FOR EXPERIMENTS WITH HUMAN TISSUE SAMPLES

Human tissue samples offer a unique opportunity to introduce an additional experimental resource on the continuum extending from immortalized tissue culture cells through mouse and primate model systems to actual treatment of infected patients. The general strategy, used most widely with palatine tonsil, rectal, and FRT mucosal tissues,[115] has been to collect tissues from uninfected patients for short-term *ex vivo* HIV-1 exposure and infection studies of events occurring in proximity to mucosal surfaces. To minimize cell emigration, cell death, and overall loss of structural integrity, tissue samples are evaluated and processed with the shortest possible delay (**Table 3**). Every patient and, consequently, every tissue sample can be expected to have unique characteristics. Experience gained from processing a wide variety of human mucosal tissue samples has led to the realization that "normal" defines a range of conditions, in which textbook quality morphology is the exception rather than the rule. This conclusion is well recognized by pathologists but, seemingly, is not fully appreciated in the wider research community. "Normal" morphology equates to a range of variance away from the idealized state and, equally importantly, epithelial surface structure and barrier characteristics may change from normal to abnormal within 50–100 μm. Every piece of human tissue is different, and we have evidence from both histological and biochemical analyses that large differences can occur in adjacent regions on the surface of a single piece of tissue.[105,107,121] These same conclusions regarding tissue variability also apply to macaques. The critical point here is that, although variability in macaque FRT morphology undoubtedly affects macaque susceptibility to SIV infection,[63,122–124] further insights into the specific events underlying HIV-1 transmission can only be derived by direct analysis of human premenopausal FRT.

A single breach in an otherwise intact epithelial barrier may be all that is required to allow initiation of primary HIV-1 infection. Independent analyses involving both HIV-1-infected

Table 3 General scheme for routine processing of human FRT tissue

Tissue stored at 4°C after processing in a surgical pathology laboratory
Tissue collected and examined macroscopically as soon as possible
Tissue photographed and evaluated—mucus removed for wet mount examination
Tissue cut and processed for organ culture infections/disruption/fixation
Infections generally initiated within 4–6 h of completion of surgery (tissue that cannot be processed without delay is generally not used for infection)
Regular monitoring of organ cultures to maintain moist mucosal surfaces
Tissues fixed and processed according to pre-determined protocols

Abbreviation: FRT, female reproductive tract.
Complete experimental control resides within the primary laboratory, including immediate design revision and/or termination in response to unexpected developments.

patients and experimentally infected female macaques support the interpretation that infection may be initiated at a single location within the FRT. Molecular techniques based on single-genome sequencing have clearly demonstrated that one or very few genomes predominate in the early stages of an expanding infection derived from a complex virus inoculum.[125–129] The same conclusion is supported by high-dose SIV vaginal infection that, at the single cell level revealed by *in situ* hybridization, frequently results in one or very few independently established foci of infection. New infections are most commonly first observed within the endocervix,[116] even though the virus inoculum would have been widely distributed across the mucosal surfaces of the macaque FRT.[130]

Microscopic analysis of appropriately stained tissue sections reveals differences in both structure and function in columnar epithelial cells within small distances across the endocervical surfaces. Structural differences include immediate proximity to leukocytes and the vasculature and lymphatics systems.[43] Functional differences in columnar epithelial cell membranes are revealed by immunocytochemistry procedures, as individual cells show cell-by-cell variability.[70]

Additional elements of variability and complexity may arise from the non-uniform distribution of commensal organisms and/or opportunistic pathogens across the FRT mucosal surfaces. The medium for *ex vivo* organ cultures usually includes antibiotics and an anti-fungal compound to prevent outgrowth of agents that were naturally present at the mucosal surface and adventitious air-borne contaminants.[105,115] Under these conditions, there is still the opportunity, or unavoidable necessity, of considering mitogenic or other stimulatory or inhibitory signals delivered by killed organisms to target human cells.

Over the time course of *ex vivo* incubations, several types of changes have been noted in FRT mucosal tissues that may affect experimental outcome. The integrity of stratified squamous epithelial surfaces is usually not well maintained as the most external cell layers rapidly detach and the entire epithelial layer may separate away, leaving only the basal cell layer at the surface of the tissue piece.[131] Conversely, endocervical tissue surfaces usually maintain cell viability with good preservation of structural integrity in organ cultures up to 7 days.[132] Endocervical columnar epithelial cells frequently secrete mucus that coats the tissue surface, and mucus probably contributes directly to retention of cell viability. In addition, a form of epithelial cell repair occurs at cut tissue surfaces with initial proliferation of highly elongated cells that create a new surface layer (PJ Southern, unpublished observations for the endocervix; epithelial surface repair for palatine tonsil samples in organ culture has been documented previously[107,121,133]). Changes in both cell composition and cell function that occur during *ex vivo* incubations may affect biochemical outcomes relating to primary HIV-1 infection, but these parameters can only be assessed with inclusion of histological analyses of tissue samples on termination of experimental incubations.[131,132]

Model systems using mixed human cell populations

During the recent development phase of human *ex vivo* organ culture systems, several other experimental resources have been created with the goal of expanding opportunities for basic HIV-1 research. Mice have been generated by transplantation technologies that maintain human adaptive immune system components including CD4+ T cells that support HIV-1 replication.[134–136] SCID-hu mice (Severe Combined Immunodeficiency-humanized)[137,138] and BLT mice (Bone Marrow, Liver, Thymus)[139,140] are not necessarily optimal for transmission studies because they retain mouse mucosal and stromal components, and natural routes of HIV-1 transmission begin with mucosal exposure. Another interesting approach has seen the development of multi-layered stratified squamous epithelial membranes (MatTek, Boston, MA) that are derived in an *ex vivo* differentiation system, using human ectocervical epithelial cells[141,142] or vaginal epithelial cells.[143] These membrane systems are notable for their highly uniform composition but, with this property, there is considerable departure from the heterogeneity that typifies human FRT mucosal surfaces. Both the reconstructed mouse systems and the differentiated ectocervical membrane system undoubtedly represent valuable technical resources and will continue to be used for HIV-1 exposure/infection studies, but key elements of these models deviate from the known specifics of community-based HIV-1 transmission.

HUMAN SEMEN AS AN ESSENTIAL EXPERIMENTAL COMPONENT

A compelling argument can be made that heterosexual HIV-1 transmission, from men to women, will almost always involve contact with semen. Therefore, the cellular and molecular processes that underlie heterosexual transmission of HIV-1 can only be fully appreciated in systems that are based on human materials and include components that are normally present in semen. Human semen has been studied for many years, primarily in terms of understanding male fertility and basic reproductive

biology.[144–147] A normal, healthy adult man releases approximately 75–100 million viable, motile spermatozoa per milliliter of semen, with a typical ejaculate volume of 2–5 ml. In addition, varying numbers of leukocytes, mainly neutrophils with some representation of macrophages and T cells, and epithelial cells are released in semen.[148–150] Leukocyte counts in semen in excess of 1 million cells per ml (a condition defined as leukocytospermia) occur with urinary tract infections and/or with large numbers of defective spermatozoa, linked to male infertility. Under conditions of leukocytospermia, the excess content of leukocytes is composed primarily of neutrophils, but individual patients with strikingly different leukocyte profiles in semen can be identified quite readily.[149,151] At present, there is only very limited understanding of the processes by which leukocytes enter the male reproductive tract and sources of HIV-1 infectivity that are shed in semen.[152,153] The cell-free component of semen, seminal plasma, is a protein-rich fluid containing high levels of proteases, esterases, DNases, and phosphatases in addition to various cytokines, complement, prostaglandins, fibronectin, and high levels of zinc ions.[154–159] Fibronectin is known to bind to gp120 on the virion surface,[160] and depending on the composition of the viral envelope, other binding interactions involving HIV-1 virions in semen may also occur.[161,162] In total, semen must therefore be recognized as a highly bioactive fluid with the potential to modify the surface of HIV-1 virions and cause transient changes in the FRT that are induced either by heterologous male cell populations and/or by the mixture of soluble mediators contacting the exposed mucosal surfaces. Additional studies, building on the foundations established for the properties of human cervical mucus,[112,163] are necessary to clarify the interactions between HIV-1 virions or HIV-1-infected cells suspended in semen and cervical mucus.

During unprotected intercourse, semen is deposited at the head of the vaginal vault in proximity to the cervical os. Spermatozoa may then migrate upward into the endocervical canal.[164] Fertilization usually occurs within the fallopian tubes, meaning that spermatozoa must ascend through the endocervical canal and traverse the endometrial surface to enter the fallopian tubes. On the basis of measurements of mobility for spermatozoa, this process could be theoretically completed within 10 min but generally may require several hours.[165] Spermatozoa can remain viable within the FRT, and complete fertilization up to 5 days after intercourse[166] and motile spermatozoa have been recovered from cervical mucus up to 7 days after intercourse.[167] In an attempt to conceptualize the details of heterosexual HIV-1 transmission, we consider the distribution and movement of spermatozoa as a surrogate marker for the potential distribution of HIV-1 infectivity across the mucosal surfaces of the FRT. Some studies have reported that spermatozoa may actually be infected with HIV-1 and/or that HIV-1 virions may bind to spermatozoa.[168,169] Although these findings may not be universally accepted,[22,170] there is a valuable outcome in forging a clear connection between HIV-1 transmission and reproductive biology. From our own work, we have demonstrated that spermatozoa and HIV-1 virions can independently bind and then penetrate beneath the surface of the stratified squamous epithelium. Surface binding and penetration of foreign cells and particles, namely spermatozoa and male leukocytes and HIV-1 virions, are likely to induce transient changes in FRT epithelial surfaces, with the potential to increase overall susceptibility to primary HIV-1 infection. Equivalent binding and penetration of spermatozoa and HIV-1 virions have also been observed at the endocervical surface, where the limited protective characteristics of the single cell layer columnar epithelium together with the common occurrence of submucosal inflammation appear to create a highly vulnerable site for primary HIV-1 infection.[70,105]

Despite the clear potential for semen to cause transient changes in FRT, there has only been limited consideration of the importance and contribution of co-factors for HIV-1 transmission that could be derived from semen. For example, virions in seminal plasma become coated with fibronectin and then may bind to epithelial cell surface integrins through fibronectin bridges in a process that resembles fibronectin-mediated bacterial binding and internalization by mammalian cells.[160,171] More recently, two independent studies have concluded that HIV-1 virion binding to DC-SIGN[172] is substantially reduced in the presence of seminal plasma, bringing into question the contribution of HIV-1 virion capture by DCs as a major mechanism for HIV-1 uptake across mucosal barriers.[173,174] In another study of physical interactions between HIV-1 virions and semen, amyloid fibrils derived from a peptide fragment of prostatic acid phosphatase in semen were shown to bind HIV-1 virions and to enhance infection of target cells.[175] On the basis of this property of influencing HIV-1 infection, amyloid fibrils have been termed "SEVI" (Semen-derived Enhancer of Virus Infection). At present, the biological significance of SEVI remains somewhat difficult to evaluate based on published work conducted *in vitro* and the apparent requirement for prolonged incubations of seminal plasma at low temperatures to trigger fibril formation.[175,176] Variable levels of SEVI activity were recently observed within a panel of normal semen donors,[177] suggesting that amyloid fibrils may be naturally present in semen and therefore available to facilitate HIV-1 transmission in the exposed FRT. The possibilities for additional interactions between HIV-1 virions and semen are further enlarged by the incorporation of host-cell constituents into the HIV-1 envelope.[161,162] Collectively, the fundamental importance of acquiring new insights into HIV-1 transmission by conducting studies in the presence of human semen is underscored both by these recent findings with SEVI and earlier studies.[178–180]

To facilitate the inclusion of semen or seminal plasma into standard experimental protocols, a series of relatively straightforward handling procedures has been developed. Viable spermatozoa and viable spermatozoa pre-labeled with the fluorescent dye CFSE have been frozen and stored in liquid nitrogen (standard viable cell-freezing protocol) and samples of undiluted cell-free seminal plasma have been stored at −80°C.[181] We have also created a consistent surrogate infectious sample by mixing a low-passage patient isolate stock of HIV-1 (HIV96-480)[107] with semen or seminal plasma obtained from normal donors. This strategy circumvents the wide variability in cellular content and

Table 4 Frequent occurrence of STIs in human populations

Transmission Documented in Semen:
HIV-1, HTLV-I, HBV, HCV, HSV-1, HSV-2, CMV, EBV, HHV-6, KSHV, HPV
Chlamydia trachomatis, Neisseria gonorrhoeae, Gardnerella vaginalis
Mollicutes sp. (Mycoplasmas)
Candida albicans
Transmission by Intimate Contact:
HSV-1, HSV-2, HPV
Haemophilus ducreyi, Gardnerella vaginalis, Chlamydia trachomatis
Neisseria gonorrhoeae, Treponema pallidum, Group B Streptococci
(*Mobiluncus*?)
Candida albicans, Trichonomas vaginalis, Schistosoma haematobium
Low-Level Commensal Infections with Potential for Pathogenic Expansion:
Staphylococcus aureus, Candida albicans
Populations of anaerobic bacteria linked to bacterial vaginosis

Interpretations and implications: Male and female reproductive tissues are highly susceptible to microbial infections. Transmission of STIs frequently involves exchange of body fluids. Polymicrobial infections and synergy between microbes may be extensively underrecognized. STI, sexually transmitted infection.

HIV-1 infectivity that has been observed in semen samples from HIV-1-infected donors.

CONCLUSIONS

Several different groups have published important findings derived from *ex vivo* FRT human organ culture systems that, cumulatively, validate the feasibility, utility, and overall importance of this experimental approach.[56–58,105,182] The potential advantages and the unavoidable limitations of the *ex vivo* systems are now quite apparent (**Table 1**), and the challenge remains to refine the experimental systems further to derive additional insights into specific events linking HIV-1 exposure and progression to primary infection. This task will undoubtedly require analytical procedures to visualize cell–cell interactions and immunological outcomes for cell populations situated in immediate proximity to human FRT mucosal surfaces. There are continuing applications for organ culture protocols in the realm of microbicide evaluation with respect to both documenting anti-HIV-1-inhibitory effects and minimizing mucosal perturbation that could inadvertently trigger inflammatory responses.[183,184] Looking further ahead, there may be opportunities to retrieve biopsy samples from vaccinated individuals, so that local innate and adaptive immune responses and resistance to HIV-1 challenge can be directly assessed by *ex vivo* organ culture. There are also potentially unlimited opportunities to add complexity back into the experimental systems, in the context of HIV-1 infection, in pursuit of a more complete reconstruction of the premenopausal FRT environment. This can be achieved with the inclusion of viable bacteria—commensal *Lactobacillus* spp. and/or reproductive tract bacterial pathogens, including *Staphylococcus aureus*, *Gardnerella vaginalis*, *Chlamydia trachomatis*, Group B Streptococci, or anaerobic bacterial populations, linked to bacterial vaginosis—exogenous viruses or even eukaryotic microbes.[185] The principal microbial agents linked to sexually transmitted infections [31,39,153,186,187] are listed in **Table 4**, and with well-defined short-term experimental goals, any of these infections can now be usefully studied in *ex vivo* FRT systems that have been described. On the basis of experiences in the Southern laboratory, most organ culture experiments contribute new information and insights relating to the human condition that were not known before initiation of the experiment.

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DISCLOSURE

The authors declared no conflict of interest.

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