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ABSTRACT

Sexually transmitted viruses infect the genital and colorectal mucosa of the partner exposed to contaminated genital secretions through a wide range of mechanisms, dictated in part by the organization of the mucosa. Because understanding the modes of entry into the organism of viruses transmitted through sexual intercourse is a necessary prerequisite to the design of treatments to block those infections, *in vitro* modeling of the transmission is essential. The aim of this review is to present the models and methodologies available for the *in vitro* study of the interactions between viruses and mucosal tissue and for the preclinical evaluation of antiviral compounds, and to point out their advantages and limitations according to the question being studied.

1. Introduction

The objective of this review is to present the methodologies available for the *in vitro* study of how sexually transmissible viruses enter the human organism and to describe their advantages and limitations according to the question to be studied. *In vitro* approaches are essential to the study of the sexual transmission of pathogens for several reasons: (i) through the use of human primary cells and human tissues, they bring us closer to the physiology and specificity of humans, thus differing from animal models, especially rodents, which cannot be directly extrapolated to humans and require cross-validation studies; (ii) *in vitro* analysis enables sequential dissection of the events leading to mucosal infection, from the mechanisms of crossing the epithelial barrier to host recognition of pathogens, induction of the local immune response, and finally infection of the target cells; iii) modeling this transmission at the mucosal level is an essential tool for developing and testing compounds aimed at preventing it; (iv) the use of human cells and tissue responds to societal ethical concerns about animal experimentation. Optimal use of these models nonetheless requires a careful determination of their advantages and their limitations.

After briefly describing the mucosal sites likely to be exposed to viruses present in genital secretions and the different mechanisms of infection, we will present a panorama of the *in vitro* study models available. Finally, we will delineate in more detail the studies conducted until now and the relevance of each model according to the event sequence under study.

2. Mucosa exposed to viruses present in genital secretions

As a general rule, the organization and specific cell composition of mucosa dictate the mechanisms of viral transmission. The types of mucosa likely to be exposed to a sexually transmissible virus are single or multiple layers of epithelial cells, more or less keratinized, that lie on a lamina propria made up of fibroblasts, immune cells, and scattered blood vessels bordered by endothelium and a deeper layer of muscle cells. A thick mucus covers mucosal epithelial cells and thus represents the first physical barrier to the exterior environment. The effectiveness of the epithelial cell barrier varies according to its thickness and organization. By secreting cytokines, chemokines, and antimicrobial compounds, epithelial cells also constitute a chemical barrier and thus play a critical role in the organism's defense against pathogens (for review (Nguyen et al., 2014)). These cells play a dual role, both responding directly to pathogens and signaling this aggression to the cells of the innate and adaptive immune systems. Specifically, mucosal

Abbreviations: BSA, Bovin Serum Albumin; CCL5, Chemokine Ligand 5; CCR7, Chemokine Receptor Type 7; CD, Cluster of Differentiation; CMV, Cytomegalovirus; DC-SIGN, Dendritic Cell-Specific ICAM-Grabbing Non-integrin; DNA, Deoxyribonucleic Acid; EDTA, Ethylenediaminetetraacetic acid; FITC, Fluorescein Isothiocyanate; GFP, Green Fluorescent Protein; HIV, Human Immunodeficiency Virus; HPV, Papillomaviruses; HSV, Herpes Simplex Virus; HTLV-1, human T-cell lymphotrophicviruse 1; IFNβ, Interferon Beta; IL, Interleukin; IRF3, Interferon Regulatory Factor 3; LDH, Lactate Dehydrogenase; MIP-1α, Macrophage Inflammatory Protein; MTT, dimethylthiazol diphenyl tetrazolium; NFκB, nuclear factor-kappa B; NK, Natural killer; PCR, Polymerase Chain Reaction; PHA, Phytohemagglutinin; PRRs, pattern recognition receptors; RNA, Ribonucleic Acid; SDF-1, Stromal cell-Derived Factor 1; SEVI, Semen-derived enhancer of Virus Infection; Th17, T Helper 17; TNFα, Tumor Necrosis Factor; VZV, Varicella-Zoster Virus; ZO-1, Zonula Occludens

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epithelial cells express, depending on their tissue origin, a repertoire of pattern recognition receptors (PRRs) that enable them to recognize the different antigenic stimuli to which they are exposed. Generally, PRR recognition of a viral or bacterial antigen leads to the secretion of a panel of proinflammatory cytokines (such as IL-6, IL-8, TNFα, and SDF-1), of β-chemokines (MIP-1α and −1β) but also of IFNβ, which plays a major role in the development of antiviral immunity (for review (Nguyen et al., 2014)).

2.1. Female genital mucosal tissue

The vagina, ectocervix, and endocervix are the principal gateways in women for viruses transmitted by semen. A stratified squamous epithelium coats the mucosa of the vagina and of the ectocervix; its thickness ranges from 15 to 30 cell layers and, for the vagina, varies during the menstrual cycle. Memory T lymphocytes, mostly CD8+, and Langerhans cells embedded in these epithelia play an important role in viral transmission, especially of the human immunodeficiency virus (HIV) (for review (Shattock and Moore, 2003)). The lamina propria of these epithelia contains resident and recirculating (CD4 + CCR7 + CD69-) memory T cells, monocyte-derived macrophages and dendritic cells, while B lymphocytes and NK cells are rarer there (Shen et al., 2009; Swaims-Kohlmeier et al., 2016; Trifonova et al., 2014). Dendritic cells are mostly myeloid, with a CD11b + CD14 + mucosal phenotype that differentiates them from conventional dendritic cells (Duluc et al., 2013; Rodriguez-Garcia et al., 2017; Shen et al., 2014a, b) and express a particular C-type lectin, DC-SIGN, which plays a primordial role in the capture and transmission of several infectious agents, including HIV and cytomegalovirus (CMV) (Trifonova et al., 2014). Typically, the mucosal concentration of immune cells is clearly lower in the vagina and the ectocervix than in the endocervix or endometrium.

A simple columnar epithelium coats the mucosa of the endocervix and the endometrium; underneath are present numerous lymphoid clusters composed of B lymphocytes surrounded by CD8+ T lymphocytes, a halo of macrophages and scattered CD4+ T lymphocytes, preferentially Th17, dendritic cells, and numerous NK cells (Nguyen et al., 2014; Rodriguez-Garcia et al., 2014, 2017). The specific expression of CD103 by dendritic cells of the endometrium may play a critical role in maintaining immune tolerance in the uterus (Rodriguez-Garcia et al., 2017; Stary et al., 2015). Unless an embryo is implanted, the endometrial mucosa is eliminated at the end of each menstrual cycle, thereby transiently enabling the deepest layers of the endometrium to be exposed to pathogenic agents.

Between the ectocervix and the endocervix lies the transformation zone, expressed histologically by an abrupt change in the epithelial surface, from stratified to simple. The mucosa of the transformation zone contains the most immune cells of any site in the female genital tract and is therefore considered to be most sensitive to infection by HIV and papillomaviruses (HPV) (Pudney et al., 2005).

2.2. Male genital mucosal tissue

The foreskin and the penile urethra are the two entryways for sexually transmitted infections in men (Pudney and Anderson, 2011). The foreskin is coated by a stratified keratinized epithelium, least keratinized on the internal face in contact with the glans (Ganor et al., 2010), in which Langerhans cells and numerous memory CD8 + T lymphocytes are embedded. The immune cells in the lamina propria are mostly macrophages and memory T cells, essentially CD8 + (Anderson et al., 2011). The penile urethra, coated by a columnar, nonkeratinized, pseudostratified epithelium, evolves toward a nonkeratinized and then keratinized stratified epithelium at the distal urethral meatus. Macrophages, memory CD8+ T lymphocytes, and Langerhans cells are embedded in the stratified epithelium. NK cells, memory CD4+ and CD8+ T lymphocytes, and naive T lymphocytes are present in the lamina propria of the pseudostratified epithelium (Nguyen et al., 2014). Most T lymphocytes in the lamina propria or infiltrating the urethral epithelium have a memory phenotype and express CD103 (αEβ7 integrin) an adhesion molecule that enables lymphocytes to adhere to epithelial cells (Pudney and Anderson, 2011). Dendritic cells are not found in the urethral mucosa.

2.3. Anal and colorectal mucosal tissue

The anal mucosa is formed by a stratified keratinized epithelium. The colorectal mucosa, located just above the anal mucosa, is formed by a simple columnar epithelium. Numerous immune cells are sparsely distributed in the lamina propria of this epithelium: CD4+ and CD8+ T lymphocytes and B lymphocytes, NK, dendritic and Langerhans cells, as well as numerous CD14+ CD68+ CD163+ DC-SIGN+ macrophages with an M2 (anti-inflammatory) phenotype. T lymphocytes population is particularly dense in area of lymphoid aggregate. T lymphocytes essentially CD8+ are additionally located sparsely intraepithelially. These numerous immune cells make this mucosa a privileged site for the entry of viruses targeting immune cells, such as HIV (Cerf-Bensussan and Guy-Grand, 1991; Isidro and Appleyard, 2016; Preza et al., 2014). Between the mucosa of the anus and that of the colon, the epithelium of the transformation zone or anorectal junction is stratified and nonkeratinized.

2.4. Modes of infection of mucosa exposed to genital secretions (Fig. 1)

Sexually transmissible viruses, such herpes simplex viruses (HSV) or papillomaviruses, can productively infect the epithelial cells that cover mucosal tissues. To propagate the infection to subepithelial targets, viruses that do not productively infect epithelial cells (*e.g.,* HIV or the human T-cell lymphotrophic viruses HTLV-1 and 2) require either impairment of the integrity of the epithelial barrier or establishment of a mechanism of infection that will override this barrier. Accordingly, the virus may reach the subepithelial target cells by: i) simple diffusion across breaches that result from micro-abrasions of the epithelial barrier during sexual intercourses or the presence of inflammation associated with a prerequisite infection of the mucosa; ii) capture of viral particles by extensions of submucosal dendritic cells, which thus participate in both submucosal and systemic viral dissemination; iii) active passage of infected cells between epithelial cells by transmigration, without impairment of the integrity of the epithelial barrier; iv) transcytosis across the epithelial barrier of viral particles, either free or from infected cells, that is, the polarized, vesicular transport of viral particles inside epithelial cells, from the apical towards the basal pole, without productive infection of the cell being crossed (for review, (Anderson et al., 2010; Shen et al., 2014a, b)).

Despite the expression of tight junctions between epithelial cells, simple epithelia are thinner and more fragile than stratified epithelia and thus more susceptible to breach formation. Inversely, fainter junctions are observed in stratified epithelia; these promote epithelial infiltration by immune cells, which may be the targets of viruses such as HIV and thus contribute to the propagation of viral infection.

The effect of hormones on mucosal tissues, their microbiota, and their exposure to genital fluids during sexual relations are all factors that can influence their infectivity (Burgener et al., 2015). For example, semen stimulates proinflammatory cytokine production by female epithelial cells and thus promotes the activation and recruitment of immune cells, which are potential targets of pathogens (Doncel et al., 2014, 2010). Mucosal exposure to genital fluids may also modulate expression of adhesion molecules expressed by epithelial cells, including the tight junction proteins such as ZO-1 and claudins (Lawrence et al., 2012; Mullin et al., 2017). Several cationic peptides present in semen increase the infectivity of HIV and CMV *in vitro* by promoting viral attachment to cells through electrostatic interactions (Munch et al., 2007; Roan et al., 2014). However, in HIV infection this effect is counteracted by CCL5, a chemokine increased in the semen of HIV-

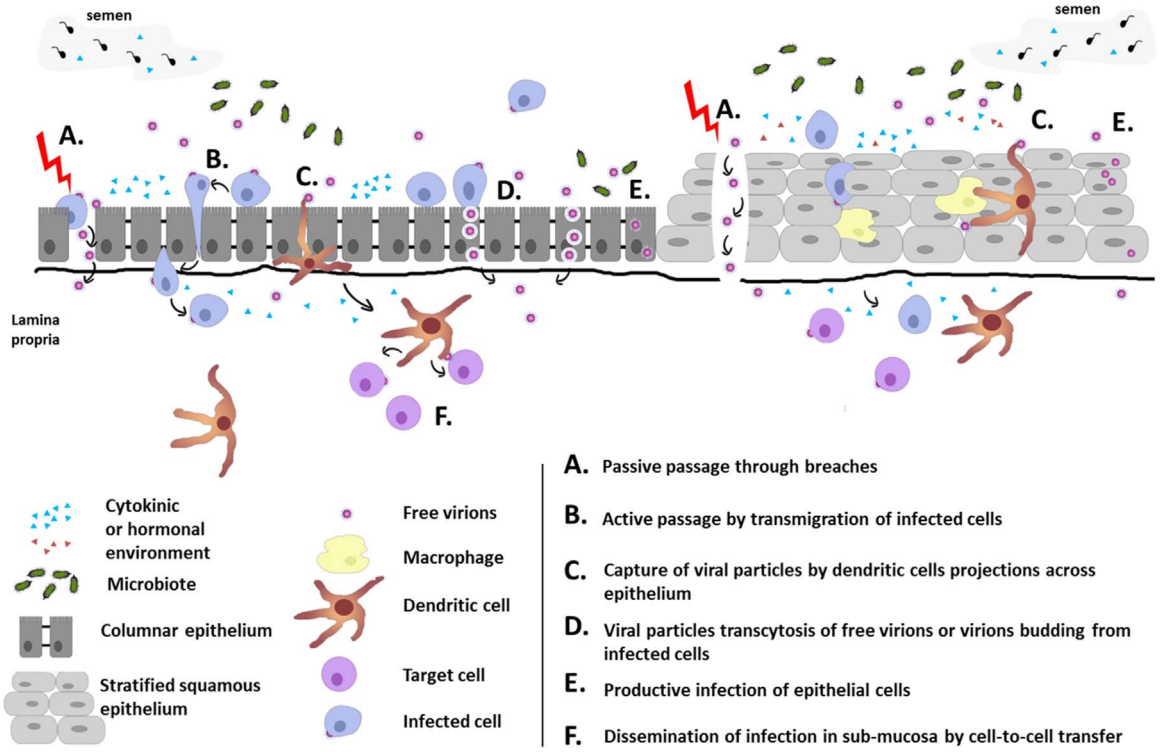

Fig. 1. Mechanisms underlying mucosal transmission of sexually transmitted virus. (A) Infected cells or viral particles may cross the epithelial barrier through breaches; (B) Infected cells may migrate between epithelial cells to infect susceptible host cells in the lamina propria (transmigration) without disrupting the barrier; (C) Sub-mucosal dendritic cells in columnar epithelia or dendritic cells embedded in stratified epithelia may capture luminal virions through their projections across the epithelia and transmit them to target subepithelial cells. (D) Viral particles transcytosis of free virions or virions budding from infected cells to infect target cells in the sub-mucosa. (E) Productive infection of epithelial cells. (F) Sub-mucosa infection dissemination by cell-to-cell transfer.

infected men and that inhibits viral entry into CD4+CCR5+ T lymphocytes (Camus et al., 2016). Moreover, other substances in semen such as clusterin and mucin 6 inhibit the HIV binding to dendritic cells mediated by DC-SIGN (Sabatte et al., 2011; Stax et al., 2009). Thus genital secretions, *via* their effects on pathogens, epithelial cells, and mucosal immune cells, can influence infection.

3. Panorama of different types of *in vitro* study models

We present below a general description of the different models that have been developed for the *in vitro* study of mechanisms of mucosal infection by sexually transmissible viruses (Fig. 2).

3.1. Cultures of immortalized epithelial cells

The cell lines used to study the infection of mucosal tissue are either epithelial cells isolated from an adenocarcinoma (*e.g.,* HeLa cervical cells) or cell lines immortalized by transduction with a retroviral vector, such as endometrial HEC-1, endocervical End1/E6E7, colorectal Caco-2. The advantage of these cell lines is that they are well characterized, standardized, and, unlike all primary models, an easily accessible biological material. Moreover, it is simpler to experiment gene silencing by siRNA or gene overexpression in cell lines than in primary cells, although the new gene editing technology CRISP/Cas9 has now greatly eased gene manipulation in primary cell culture.

Some epithelial cell lines allow the formation of a monolayer of fully

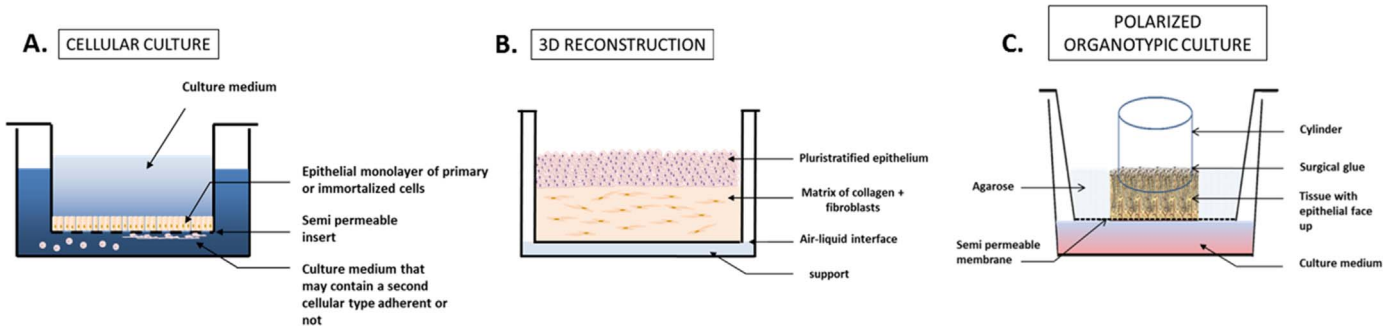

Fig. 2. *In vitro* culture systems of columnar and stratified mucosal epithelia. (A) Cellular culture system: Primary or immortalized cells are plated on a semi permeable insert. When seeded in specific conditions, they may allow the formation of a monolayer of fully differentiated and polarized epithelial cells. Primary or immortalized cells, adherent or not, can be added in basal supernatant. (B) 3D reconstructed system: Primary or immortalized cells are cultured several days on a matrix of collagen and fibroblasts feeder cells intending to mimic the mucosal lamina propria. The matrix/epithelial cell assemblage is then placed at the air-liquid interface or is submerged. The epithelial cells then stratify and differentiate into an epithelium mimicking that of the tissue of origin. Improved models integrate the immune cells physiologically embedded among these epithelial cells. (C) Polarized organotypic culture system: Tissue fragment is placed, apical face up, on porous membrane (or gelfoam) in a two-chamber system and cultured at the air-liquid interface. The explant is surrounded by 3% agarose and/or a cylinder fixed by surgical glue on epithelial face allowing the polarized deposit of compounds/virus.

differentiated and polarized epithelial cells. This generally requires culture for 8–10 days at confluence on semi-permeable inserts, coated or not by a matrigel-type protein support. When the cells reach confluence, they begin to differentiate and become polarized. The polarization of the epithelial cell culture is verified by measuring transepithelial resistance with an ohmmeter, visualizing tight junction expression, or testing the extracellular passage of high molecular weight compounds, such as dextran.

3.2. Primary cultures of epithelial cells

The use of primary cells, isolated from fresh tissues, eliminate potential artefacts associated with the use of immortalized cultures. Epithelial cells are isolated by enzymatic digestion of the tissue, sometimes associated with mechanical digestion (for review (Kaushic et al., 2011)). Some isolated epithelial cells can be cultivated in polarized monolayers and kept in culture for around two weeks. This polarization can be more difficult to obtain than in cell lines (Bomsel and Alfsen, 2003), since the purification of primary cells can compromise the function of the epithelial barrier and prevent the reformation of the tight junctions necessary for polarization.

3.3. Co-culture of epithelial cells with cells from genital secretions or from the lamina propria

Classically, in a two-chamber culture system, a second cell type is added to a confluent culture of primary epithelial cells or an epithelial line (polarized or not) grown on a semipermeable insert. Co-culture systems for epithelial cells and leukocytes (either primary cells or cell lines) added apically in the insert, have thus been developed to mimic the contacts between the epithelial cells and the cells present in the donor's secretions (Lawrence et al., 2012). There are also systems where the two types of co-cultured cells are adherent, *e.g.* epithelial and dendritic cells to study interaction with submucosal immune cells (Cavarelli et al., 2013). In the latter case, after polarization of the epithelial cells on the insert, the culture chamber is turned over and the dendritic cells deposited on the opposite side of the insert. After several hours, when the dendritic cells have adhered to the insert, the system is returned to its initial position, with the epithelial cells resting on the basal face of the insert and the adherent dendritic cells under the insert and thus under the basal face of epithelial cells.

3.4. 3D reconstructed stratified mucosa

3D reconstructions aim to mimic the characteristics of the stratified mucosal epithelia of the vagina, ectocervix, foreskin, or urethra. These models involve culturing primary or immortalized epithelial cells for several days on a matrix of collagen and fibroblast feeder cells, before placing at the air-liquid interface or submerged. The epithelial cells then stratify and differentiate into an epithelium mimicking that of the tissue of origin (for review (Andrei, 2006)). Improved models integrate the immune cells physiologically embedded among these epithelial cells, such as dendritic cells, Langerhans cells, or macrophages, which are added at the moment of the matrix/epithelial cell co-culture (Bouschbacher et al., 2008; Ganor et al., 2013, 2010; Sivard et al., 2004). It generally takes 5–8 weeks to obtain a complete reconstruction with a polarized stratified epithelium. Compared with organotypic cultures (see next paragraph), these 3D reconstructed models have homogeneous epithelial barriers, and the tissue architecture can be maintained intact long enough to study their infection. Nonetheless, they lack the physiological value of organotypic cultures because they are composed of a mixture of primary and immortalized cell populations, often derived from different sources.

3.5. Organotypic cultures

Organotypic cultures are the most integrative and physiological model available today for researchers to study the mechanisms of the sexual transmission of viruses *in vitro*. Organotypic culture models exist for foreskin, ectocervix, endocervix, vagina, and colorectal tissue. Fragments of tissue containing both epithelium and submucosa are obtained after surgery and cut into small blocks of several mm^2 each to avoid anoxia at the center. The tissue is cultured most often at the air-liquid interface on porous membranes of polycarbonate or collagen in two-chamber systems or on gelatin sponges (gelfoam). To mimic the mechanisms of viral dissemination most accurately the tissue can be oriented with the epithelial face up and the submucosal face in contact with the membrane or gelfoam and the explant surrounded by agarose (Collins et al., 2000; Kolodkin-Gal et al., 2013; Maher et al., 2005; Tugizov et al., 2011) or by a cylinder attached with surgical glue to the epithelial face of the tissue (Cavarelli et al., 2013; Ganor et al., 2013, 2010; Shen et al., 2010, 2009; Tsilingiri and Rescigno, 2012), so that only the epithelial face is exposed to the virus.

The major advantage of organotypic cultures is that they preserve the original tissue architecture. Nonetheless, the maintenance of this architecture may be very short, due to limited survival of some cell types which are less well "fed" than in the 2D culture system, either because of more restricted access to nutrients, or because the culture medium used must be a compromise between the needs of the different cell types. The period that the explant's epithelial barrier integrity remains intact therefore varies quite substantially according to tissue origin and culture medium enrichment. As a general rule, the most superficial strata of stratified epithelia are lost by 24–48 h after the culture starts (Collins et al., 2000; Maher et al., 2005). Moreover, the immune cells embedded in these epithelia begin to emigrate outside the tissue almost immediately. Concerning the mucosa with simple epithelium, it exists a hide heterogeneity depending on tissue: endocervical epithelia remain intact in the culture for 4–6 days (Maher et al., 2005), but colon epithelia are much more fragile, remaining intact for no more than 6 h of culture in standard conditions (conventional incubator) (Cavarelli et al., 2013; Fletcher et al., 2006; Kolodkin-Gal et al., 2013) and 24 h in an O_2 enriched atmosphere (Dezzutti et al., 2014). In all cases, the integrity of the tissue depends on extremely rapid culturing after the surgical excision. Finally, an important limitation to the use of all primary models is the supply of human tissue, which can be rare or irregular, and submitted to agreement by ethic committees.

4. Choice of models according to the questions to be studied

Through examples illustrating results obtained from various models, this section seeks to highlight the advantages and limitations of each of these models for studying the different stages of mucosal transmission of pathogens.

4.1. Study of the viral source in genital secretions

Contamination of genital secretions by viral particles and/or infected cells is responsible for the transmission of the virus to the mucosa of the sexual partner. One unresolved question under study, however, is how the virus reaches the genital secretions. This question is particularly pregnant for semen, which is an important vector for many viruses. It was long thought that the viruses present in the semen come from the passive distribution of viral particles and infected cells from the circulating blood, but it has now been established that this is not the only source. Phylogenetic analyses in men with HIV have showed that around 60% of them have viral populations in the semen that are distinct from those in their blood (Houzet et al., 2014). Another issue concerns viral persistence in semen despite negative or undetectable viremia. HIV persists in the semen of some patients receiving effective

antiretroviral treatment, and the Ebola and Zika viruses can last for several months in the semen of men who have recovered. These facts indicate the existence of local sources, which must be elucidated to determine the origin of these viruses in semen. *Ex vivo* models of human tissue are extremely useful for testing semen-producing organs permissiveness to viruses, given that it is impossible to study the organs of the genital tract of infected men. We thus demonstrated *ex vivo* infection by HIV with explants of the human testes, prostate, and seminal vesicles in organotypic culture models and identified the types of cells infected (Deleage et al., 2011; Le Tortorec et al., 2008; Roulet et al., 2006). It is nonetheless important to validate results obtained *ex vivo* by *in vivo* approaches in animals, which also enable the simultaneous study of the infection of local sources and of the semen (Matusali et al., 2015).

4.2. Study of the mechanisms of infection of the epithelial barrier

A polarized primary culture of epithelial cells is an interesting model for studying infection of simple epithelia. MacDonald *et al.* and Nazli *et al.* developed polarized primary cultures of endometrial and endocervical epithelial cells to study their infection by HSV-2 (MacDonald et al., 2007; Nazli et al., 2009). A polarized monolayer was exposed to the virus, and the cells' permissiveness to infection assessed by measuring viral release in the basal supernatant and microscopic observation of infected cells. This simplified model allowed them to study the direct effect of a variety of factors that might modify epithelial cell susceptibility to infection, such as variations in sex hormone levels (progesterone) or antiviral compounds (MacDonald et al., 2007; Nazli et al., 2009).

Nonetheless, the use of genital epithelial cells in primary cultures or from cell lines has produced divergent results for HIV infection (Greenhead et al., 2000; Howell et al., 1997). Based on *in vivo* studies of simian models, the current consensus is that HIV does not productively infect the epithelial cells of either vaginal or rectal mucosa (Hu et al., 2000; Miller and Hu, 1999; Ribeiro Dos Santos et al., 2011; Salle et al., 2010). The discordant results about the productivity of infection for the same primary cell or cell line cultures may be associated with variations in the infection protocol (viral strain, quantity of inoculated virus, and time of exposure), primary cell isolation process (loss of membrane receptors for virus needed for virus entry during the tissue dissociation), and the degree of cell polarization. More broadly, it must be borne in mind that immortalized cells do not preserve all the morphologic and biochemical characteristics of the tissue's primary cells. Transformed cell lines differ greatly from primary cells in their lipid and protein composition and their glycosylation profile (Bomsel and Alfsen, 2003). These differences can influence or even modify the mechanisms of viral entry and cell infection, compared with the situation *in vivo*. Accordingly, although essential to the study of the mechanisms of infection and the passage of different viruses, the simplified models of isolated culture of primary epithelial cells or of cell lines require validations in more integrated models.

3D reconstructed culture models have improved our understanding of the early phases of HSV infections in stratified genital epithelia (Meyers et al., 2003; Visalli et al., 1997), where the expression program of the epithelial cells differs according to the position of their stratum. For example, using a 3D culture model of primary epithelial cells of the foreskin and cervix on a matrix of collagen containing a murine fibroblast cell line, Visalli *et al.* showed that productive HSV-1 infection occurred exclusively in the most basal layers of the epithelium. 3D culture models of genital epithelial cells also enabled considerable progress in the study of HPV infections, whose replication cycle depends strongly on the differentiation of the host epithelial tissue. Epithelial cell infection is reproduced by seeding epithelial cells from biopsies of infected patients, or by transfection of epithelial cells with the HPV genomic DNA, or finally with HPV immortalized epithelial cell lines (for review (Bodily et al., 2006)). In the latter case, differentiation of the epithelium leads to the formation of different grades of neoplasms, similar to that observed *in vivo,* and can thus be used to study cancer progression (*e.g.,* by studying the capacity of mononuclear allogenic blood cells to migrate in a preneoplastic or neoplastic epithelium). This model also makes it possible to reproduce the different stages of the HPV life cycle, including episomal maintenance of the viral genome, genome amplification, and differentiation, which depends on the expression of viral genes. It thus enabled the first *in vitro* production of HPV virions (Dollard et al., 1992; Meyers et al., 1992). Several studies have thus used this model to test the effectiveness of compounds for treating HPV infection (for review (Andrei, 2006)). Thanks to a more integrative model of organotypic cultures of ecto- and endocervical explants, Horbul *et al.* demonstrated that HSV-2 infection of genital epithelial cells is cytolytic, induces inflammation of the mucosal tissue, and thereby promotes infection of the genital mucosa by HIV-1 (Horbul et al., 2011). A considerable limitation of this type of model for studying infection of a stratified epithelium is the loss of the stratification after a few days (*e.g.,* < 6 days for the ectocervical epithelium in the study by Horbul et al.).

4.3. Analysis of mechanisms for crossing the epithelial barrier in the absence of productive infection of epithelial cells

Numerous sexually transmissible viruses can cross the epithelial barrier without infecting it productively. As pointed out above (Section 2.4 on the mode of infection of mucosa exposed to genital secretions), these viruses use diverse strategies to reach submucosal target cells. In this section, we will look at the *in vitro* models developed to study these different mechanisms for crossing the epithelial barrier.

4.3.1. Analysis of the formation of epithelial breaches

The most common and easiest access for a virus or infected cells to subepithelial target cells is the infiltration through breaches in the epithelium. These can be induced mechanically by sexual intercourses, or may result from a pre-existing genital infection, or be generated by the virus itself. Inflammation of the mucosa induced by a bacterial or viral infection results in an influx of immune cells and proinflammatory cytokines, which can impair the epithelial barrier and thereby render the subepithelial mucosal cells accessible to other pathogenic agents. Some viruses, such as herpesvirus, directly affect epithelial cells by inducing herpetic ulcerations. Nazli *et al.*, studied the capacity of HIV to engender breaches (Nazli et al., 2010, 2013), using a model of polarized primary epithelial cells from the endometrium and cervix, cultured on matrigel, and polarized T84 cells (intestinal cell line). They showed that exposure to HIV particles impaired the integrity of these epithelial monolayers, as demonstrated by a decrease in transepithelial resistance, a loss of junction protein expression, and greater permeability of the epithelial barrier to dextran. These simplified models of the epithelial barrier made it possible to understand the mechanism of this impairment, and neutralization experiments demonstrated that the virus envelope protein (gp120) generates impairment of the epithelial barrier by inducing epithelial cells to secrete TNFα (Nazli et al., 2010). As in all *in vitro* studies, it is also important to consider the dose and strain of virus used in interpreting the results, to determine if they correspond to a realistic exposure.

4.3.2. Analysis of the capture of viral particles by mucosal immune cells

Another means for pathogens to gain access to subepithelial target cells is to be captured by the dendritic cells or Langerhans cells embedded in the stratified epithelia or simply present at the basal pole of the simple epithelia.

To study this phenomenon in columnar epithelium, Cavarelli et al. (2013) developed two models. The first model consisted of a co-culture on a semipermeable insert of a polarized colorectal epithelial cell line, Caco-2, and dendritic cells cultured under the insert, at the basal pole of the epithelial cells (Fig. 2 describes the set-up of such a system). The virus is then deposited at the apical surface of the epithelial cells. After

several hours of exposure, the epithelial barrier is observed by confocal microscopy, which allows in-depth visualization of the cell layer. This model made it possible to show that HIV induces dendritic cells to migrate towards the apical side of the epithelium. Given that the migrating dendritic cells were isolated from blood rather than a tissue, the physiological relevance of this simplified model may be questionable. Nonetheless, these observations were validated with a more integrative model of a colorectal tissue explant culture, in which the polarized exposure of the colorectal epithelium to HIV particles also induced mucosal antigen-presenting cells to be attracted toward the apical pole of the epithelial cells and to capture viral particles (Cavarelli et al., 2013).

To analyze how dendritic cells embedded in the stratified epithelia are involved in viral transmission, Ganor et al. (2010) developed an organotypic foreskin mucosal culture. It enabled the authors to observe with fluorescence microscopy that polarized exposure of the internal epithelial mucosa of the foreskin to blood leukocytes strongly infected by an HIV-1 strain induced rapid modification of the spatial distribution of intraepithelial Langerhans cells, which moved closer to the epithelial surface; it also induced the formation of Langerhans cell–T-cell conjugates at the level where the viral particles were observed. Nonetheless, the major limitation of stratified organotypic explant models is their rapid loss of the tissue's structural and morphologic integrity, together with the migration of the intraepithelial immune cells outside the explant several hours later. It then becomes impossible to distinguish between the migrant cells of intraepithelial origin and the immune cells of the stroma. The observation of later infection events therefore requires the development of other models, more stable but less close to *in vivo* conditions. 3D reconstructed models of stratified mucosal tissue of the vagina (Bouschbacher et al., 2008; Sivard et al., 2004) and the foreskin (Ganor et al., 2010) into which Langerhans cells were integrated are particularly appropriate for this purpose and allow a longer-term view of the role of dendritic cells in the establishment of this infection in these mucosal tissues. For example, Sivard *et al.* integrated into their 3D reconstructed model of vaginal mucosa some Langerhans cells, differentiated from CD34+ hematopoietic progenitor cells isolated from umbilical cord (Sivard et al., 2004). In this system, the authors were able to show, 48 h post-infection, the presence of viral DNA in the Langerhans cells as well as the complete inhibition of their infection in the presence of a reverse transcriptase inhibitor.

Hladik et al. (2007) developed an original model of culture of vaginal epithelial sheets (Ballweber et al., 2011), by separating the epithelial strata from the lamina propria by microdissection and EDTA treatment. The major interest of this model is that it enables the targeted study of the infection of intraepithelial immune cells without having to deal with the problem of immune cell emigration out of the tissue, since in the absent of submucosa, the only cells to emigrate come from the epithelial layer. Hladik et al. (2007) were thus able to observe by confocal microscopy the attachment and entry of viral particles into the CD4+ T lymphocytes and intraepithelial Langerhans cells of the vaginal mucosa. Several years later, this team improved the system by polarizing the infection to the apical face of the epithelial sheet. By showing that the proviral DNA was not integrated, they demonstrated that the intraepithelial Langerhans cells are not capable of productive infection by HIV, but do play a role in viral dissemination, as shown by the infection tests in a co-culture of CD4+ T lymphocytes (Ballweber et al., 2011).

4.3.3. Analysis of viral passage by transcytosis

Transcytosis is the polarized transport of virus into the interior of epithelial cells, from the apical to the basal pole, without productive infection of the host cell. The virus endocytosed by the epithelial cells can come from a free virus source or be viral particles released by infected cells that form virological synapses at the apical pole of epithelial cells. This mechanism of viral transmission in the genital or colorectal mucosa was described for HIV *in vitro* for the first time by Bomsel (1997). These authors, and other teams, have confirmed passage through the epithelium by transcytosis for HIV and other retroviruses in other *in vitro* or *ex vivo* models, but it has never been demonstrated *in vivo* (Alfsen et al., 2005; Bobardt et al., 2007; Bomsel et al., 1998, 2007; Hocini et al., 2001; Martin-Latil et al., 2012; Shen et al., 2010; Stoddard et al., 2009). HIV transcytosis has thus been shown in polarized cultures of immortalized epithelial cell monolayers derived from the intestines (I407), colon (HT29, Caco-2), and endometrium (HEC1) as well as in polarized cultures of primary vaginal and endocervical cells in monolayers, exposed either to HIV viral particles or to HIV-infected leukocytes during a period too short for the productive infection of epithelial cells. The passage of the virus through the epithelial barrier was demonstrated as early as 30 min after exposure, by measuring p24 viral proteins in the supernatant of the basal culture, by infectivity tests of reporter cells, and by observation of cell infection by electron microscopy. The viral particle internalization kinetics was assessed by measuring the p24 viral proteins in the lysed epithelial cells. Viral passage was inhibited at 4 °C and by irreversible microtubule depolymerization. Demonstration of the transcytosis of viral particles imperatively requires ensuring that the epithelial layer is sealed tight, by verifying the maintenance of transepithelial resistance, the absence of paracellular passage of extracellular markers (*e.g.,* [^{14}C]inulin or FITC-BSA) and the continued expression of tight junction proteins such as ZO-1, to prevent the passage of viral particles by a paracellular mechanism. Despite several discordances linked to the use of different infection protocols (virus concentration and time of viral exposure), these studies allow two conclusions: 1) the effectiveness of transcytosis is greatest when the epithelial cells are exposed to infected cells rather than to free viral particles, and 2) virological synapses form between infected leukocytes and epithelial cells (Alfsen et al., 2005; Bomsel, 1997; Hocini et al., 2001). Although this viral transmission mechanism has been clearly shown *in vitro* in monostratified cell culture models, its existence *in vivo,* or at the very least, in more physiological *ex vivo* models, remains to be demonstrated.

The confirmation of transcytosis in more integrative model of mucosal explants, as suggested by the results of Shen et al. (2010, 2009) in cultures of rectal or vaginal explants, is complicated by the possibility of viral transport by "Trojan horse" cells, either present in the mucosa or embedded between the epithelial cells, such as macrophages or dendritic cells. This bias can be avoided by the use of integrative models less close to the *in vivo* model but better controlled, such as 3D reconstruction models, where the cell types present can be selected according to the study question. Bouschbacher et al. (2008) thus showed that HIV particles do not pass through the stratified vaginal epithelium in a 3D reconstructed model, with or without Langerhans cells embedded in the epithelium. Using a 3D culture model of an ectocervical epithelial sheet exposed in a polarized manner either to free HIV particles or to infected cells, Greenhead et al. (2000) did not detect infection event in either the culture supernatant or the epithelial cells. These 2 studies therefore suggest the absence of HIV transcytosis across the stratified genital epithelia. Nonetheless, we cannot rule out the possibility that the stages of enzymatic digestion necessary to establish these models might engender the loss of expression of some membrane markers necessary for the attachment and internalization of viral particles.

4.3.4. Analysis of the transmigration of infected cells across the epithelial barrier

Transmigration is the active passage of infected cells between the epithelial cells without the barrier rupturing. The simplest models, which are also the fastest to set up for a study, are simple polarized epithelial cell lines (Carreno et al., 2002; Chancey et al., 2006; Lawrence et al., 2012). Accordingly, Lawrence et al. (2012) demonstrated on a polarized monolayer of immortalized endometrial cells (HEC-1) that monocytes and, to a lesser extent, CD4+ T lymphocytes, are capable of transmigration. They deposited monocytes or

lymphocytes isolated from blood, infected *in vitro* and labeled, on polarized epithelial monolayers and assessed transmigration 24 h later by scoring the number of labeled cells recovered in the system's basal supernatant or by measuring the fluorescence intensity of this supernatant. The major advantage of this simplified model of polarized immortalized epithelial cells is that it allows easy apprehension of the molecular mechanisms and environmental factors involved in the adhesion of transmigrant cells to epithelial cells and their passage to the subepithelial mucosa. Thus Lawrence *et al.* showed in their polarized HEC-1 model that seminal fluid inhibits leukocyte transmigration across the epithelial barrier. This effect is associated with increased expression of ZO-1 junction proteins between epithelial cells and greater leukocyte capacity to adhere to epithelial cells (Lawrence et al., 2012). Nonetheless, although this simplified model is both a good approach and a useful method of molecular validation, it remains somewhat distant from the *in vivo* situation and glosses over the influence of the tissue context on epithelial cell expression of adhesion molecules.

In the more physiological explant model, the transmigrating cells labeled by a fluorochrome and deposited on the epithelial apical surface, can be pursued in the tissue by fluorescent microscopy or flow cytometry after enzymatic tissue dissociation (Kolodkin-Gal et al., 2013; Maher et al., 2005). Its principal limitation is that it can keep the epithelial barrier intact for only a short period, which varies by tissue origin: around 24 h for colorectal, 48 h for ectocervical, and 4–6 days for endocervical tissue, depending on the study and culture conditions. The use of this model to observe transmigration events therefore requires that the researcher rigorously ensure that the integrity of the epithelial barrier is preserved for the time necessary for transmigration (*e.g.,* verification that labeled extracellular molecules have not passed through). Accordingly, in 3D models of human ectocervical and endocervical explant cultures, Maher et al. (2005) used confocal microscopy to observe the infiltration of seminal leukocytes into the most external layers of the stratified ectocervical epithelium, but no infiltration into the endocervical epithelium; instead the seminal cells were retained in the mucosal secretions, which thus seem to play a protective role against infection in this model.

4.4. Study of the infection of subepithelial target cells and of response to mucosal infection

Beyond the mechanisms for crossing the epithelial barrier, it is primordial for developing prevention strategies to understand how the infection settles into the mucosa (analysis of its target cells, their proportion, location in the tissue, and susceptibility to infection) and the consequences of this infection (cytopathic effect, establishment of a local immune response).

The explant model, polarized or not, is a model of choice for studying mucosal cells' susceptibility to the infection, to the extent that the original tissue context and cytokine environment present *in vivo* can be preserved for the time necessary to observe this infection. We cite here, non-exhaustively, several explant models developed to analyze the permissiveness to infection of mucosal cells of the female genital tract (Collins et al., 2000; Fox-Canale et al., 2007; Greenhead et al., 2000; Maher et al., 2005; Patterson et al., 2002; Shen et al., 2009), the male genital tract (Fischetti et al., 2009; Ganor et al., 2013; Patterson et al., 2002), or the colorectum (Fletcher et al., 2006; Kolodkin-Gal et al., 2013). In these studies, tissue infection is determined by quantitative PCR measurement of the viral genome copies number in the tissue (Fletcher et al., 2006; Patterson et al., 2002; Shen et al., 2009) or the measurement of viral proteins released into the culture supernatant (*e.g.,* p24 for HIV) (Fischetti et al., 2009; Fletcher et al., 2006; Ganor et al., 2013; Greenhead et al., 2000). The infected cell types can be identified by flow cytometric analysis after enzymatic dissociation of the tissue (Fox-Canale et al., 2007; Kolodkin-Gal et al., 2013), immunohistochemical co-labeling of cellular and viral protein markers (Fox-Canale et al., 2007; Greenhead et al., 2000; Maher et al., 2005; Shen et al., 2009), or detection of the viral genome in the mucosa cells by *in situ* hybridization combined with immunohistochemical labeling to identify infected cells (Patterson et al., 2002; Collins et al., 2000; Deleage et al., 2011; Ganor et al., 2013, 2010; Le Tortorec et al., 2008). Their identification can also be facilitated by inoculating the tissue with a modified virus expressing a green fluorescent protein (*e.g.,* CMV-GFP (Fox-Canale et al., 2007)). In this model, it is possible to mimic immune activation of the mucosal immune cells by adding PHA and IL-2 to the culture medium for the entire culture period (Fischetti et al., 2009; Greenhead et al., 2000), thereby increasing the infectivity of the mucosal cells according to the tropism of the viral strain (Greenhead et al., 2000). The analysis of the mucosal cells' permissiveness to infection in the explant model mandates verification that the virus's potential target cells are adequately maintained along the culture. Observation of tissue infection by some viruses, such as CMV, requires maintaining the culture up to 2 weeks (Fox-Canale et al., 2007). Moreover, some cell types, especially Langerhans and dendritic cells, tend to migrate outside the explant (Fischetti et al., 2009); it can thus be necessary to collect these cells to analyze their infection.

In nonpolarized explant models (Fischetti et al., 2009; Fletcher et al., 2006; Fox-Canale et al., 2007; Greenhead et al., 2000), infection of subepithelial mucosal cells is facilitated by viral inoculation of the explants during immersion and is not limited to the epithelial face of the tissue. The major advantage of these nonpolarized explant models is that they allow researchers to focus on early mucosal infection events in an integrative model; they can bypass the stage of epithelial barrier passage that requires more complex models to maintain the barrier integrity. Recently, these models were used to assess the effect of hormones in the female genital tract on its permissiveness to HIV infection (Rollenhagen and Asin, 2011; Saba et al., 2010, 2013). These models also make it possible to analyze the infection-induced immune activation, by measuring the cytokines produced by the tissue and released in the culture supernatant or by determining mucosal immune cell phenotypes by flow cytometry or immunohistochemistry, and to test the cytopathic effect of the virus on the mucosa (Fischetti et al., 2009). As long as they are used within the limits of their validity, the models of polarized explants, in which viral inoculation is limited to their epithelial surfaces, provide a more physiological response about capacity to transmit infection to mucosal cells (Ganor et al., 2010; Collins et al., 2000; Kolodkin-Gal et al., 2013; Maher et al., 2005; Patterson et al., 2002; Shen et al., 2009). For example, Shen *et al.* used polarized explant models of vaginal and intestinal mucosal tissue to study the permissiveness to HIV of mucosal macrophages. While intestinal macrophages remained insensitive to the infection, it was detected in the vaginal macrophages after as little as 30 min of exposure to an R5 strain, and this difference in permissiveness was linked to a phenotypic difference between these cells in these two different tissues (Shen et al., 2009). Nonetheless, as we mentioned above, the integrity of the epithelium can only be maintained for a limited time, especially that of the simple epithelia of the colon and must therefore be systematically validated.

The reproducibility of results obtained by the explant model is often complicated by interdonor variability of tissues in terms, for example, of hormonal and inflammatory status, history of sexually transmitted diseases, or differences of proportions in the target cell populations between the different tissue fragments.

4.5. In vitro testing of antiviral strategies

In vitro and *ex vivo* models of mucosa exposed to sexually transmissible viruses are an indispensable tool for preclinical evaluation of the safety, efficacy, and formulation of local antiviral strategies. It is essential to ensure that the substances tested do not induce tissue toxicity that might weaken the epithelial barrier or induce mucosal inflammation. It is also important to test the effectiveness of antiviral agents in models that can mimic the physiological conditions during which the risk of infection is greatest, that is, when the epithelial

barrier is compromised or in inflammatory situations.

Most of the studies thus far conducted have tested HIV compounds in culture models of primary epithelial cells or cell lines or of reconstructed 3D mucosal epithelium or tissue explants. The limitations and advantages of these different models remain the same as they are for analyzing viral transmission or mucosal cells' permissiveness to an infection. Three D reconstructed epithelial mucosa culture models are particularly appropriate for testing antiviral compounds against viruses, such as alpha-herpesviruses or HPV, that infect epithelial cells (for review (Andrei, 2006)) and for which no animal model are available.

Explants of cervical, vaginal, colorectal, and penile tissue are models of choice for testing the anti-HIV activity of microbicides (references in review (Dezzutti, 2015)). These models allowed researchers to show that the prevention of HIV-1 infection in the mucosa by different viral entry inhibitors and non-nucleoside inhibitors requires a dose several log10 higher than the doses required in traditional *in vitro* tests with reporter cells (Fischetti et al., 2009; Fletcher et al., 2009; Herrera et al., 2009, 2011).

Polarized explants present the clear advantage of enabling an assessment of the efficacy of compounds, formulated most often in gel, with an epithelial application that thus mimics the natural entry route of the substances tested (Abner et al., 2005; Cummins et al., 2007). They also allow testing the effect of genital fluids (semen and vaginal secretions) on the distribution and effectiveness of drugs to be tested.

In recent years, another original mucosa culture model has been developed and used in clinical trials. This *ex vivo* challenge model consists in putting into culture colorectal, cervical, or vaginal biopsy samples from participants who assiduously used an antiviral gel or vaginal ring over a given period. The biopsy sample was then infected *ex vivo* by HIV to assess the effectiveness of the microbicide treatment (for reference, see (Dezzutti, 2015)). Setting up these trials demands good logistic coordination between the clinic and the laboratory, but enables a more physiological evaluation of microbicide effectiveness.

5. Conclusion

The numerous *in vitro* models of genital or colorectal mucosa have provided vital information about the mechanisms by which sexually transmissible viruses infect these mucosal tissues. This variety of models is justified by the multiple mechanisms that can lead to mucosal infection, each model being most appropriate for the study of one mechanism in particular. By their simplicity and flexibility of construction (*e.g.,* variety of cell types involved, control of strains and viral dose, and inclusion of environmental factors), these models have made it possible apprehend the role of each cell type in viral transmission and to dissect the innate immune response to the infection as well as the influence of genital fluids (vaginal secretions and semen) and hormonal status on its establishment. Today they are also indissociable from *in vivo* studies for the elaboration of new strategies for local prevention. These *in vitro* models deserve greater use, especially to assess the impact of local microbiota on the infectivity of mucosal tissue. Several clinical studies indicate that modification of the vaginal or rectal microbiota, called dysbiosis, influences the risk of transmission of sexually transmitted diseases, with in particular an increase of more than 60% in the risk of transmission in the presence of a microbiota characteristic of bacterial vaginosis (for review (Burgener et al., 2015)). Recently, a 3D reconstructed epithelial model was developed that simultaneously enabled colonization by bacteria of the vaginal microbiota and study of the transmission of sexually transmissible pathogens (Pyles et al., 2014).

Most recently, methods of 3D bioprinting have attracted the attention of interest by researchers in tissue engineering. This bioprinting uses the technologies of computer-assisted printing to place — layer by layer or point by point — cells, components of the extracellular matrix, growth factors, *etc.,* thus enabling the arrangement and assembly of live biological structures in 2D or 3D with high reproducibility and repeatability. This new technology, which is a major opportunity for regenerative and reparative medicine, may also allow the manufacture of tissue models, such as highly controllable and reproducible genital and colorectal mucosa for pharmacological testing of the activity of antiviral compounds (for review (Fricain et al., 2017)). Although the bioprinting tissue models thus far developed do not yet satisfactorily reproduce tissue complexity (Fricain et al., 2017), it is probable that the development of bioprinting may in time supplant most existing *in vitro* models.

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