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Mucosal HIV transmission and vaccination strategies through oral compared to vaginal and rectal routes

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Abstract

Importance of the field—There are currently over thirty million people infected with HIV and there are no vaccines available to prevent HIV infections or disease. The genitourinary, rectal and oral mucosa are the mucosal HIV transmission routes. An effective vaccine that can induce both systemic and local mucosal immunity is generally accepted as a major means of protection against mucosal HIV transmission and AIDS.

What the reader will gain—Structure and cells that comprise the oral, vaginal and rectal mucosa pertaining to HIV transmission and vaccination strategies through each mucosal route to prevent mucosal and systemic infection will be discussed.

Areas covered in this review—Covering publications from 1980's through 2010, mucosal transmission of HIV and current and previous approaches to vaccinations are discussed.

Take home message—Although oral transmission of HIV is far less common than vaginal and rectal transmissions, infections through this route do occur through oral sex as well as vertically from mother to child. Mucosal vaccination strategies against oral and other mucosal HIV transmissions are under intense research but the lack of consensus on immune correlates of protection and lack of safe and effective mucosal adjuvants and delivery systems hamper progress towards a licensed vaccine.

Keywords

HIV; mucosa; vagina; rectum; oral; vaccine

1. Introduction

Heterosexual and homosexual transmission of HIV across epithelial cells of the genitourinary and rectal mucosa, are among the most common routes of HIV transmission [1]. Infection with HIV and the transpiring disease called AIDS has claimed millions of lives and currently an estimated 33 million humans are infected with HIV. HIV not only is transmitted mainly through mucosal membranes, it also exerts its pathogenic effects at mucosal surfaces of the gastrointestinal, and perhaps genitourinary and respiratory tracts, early on regardless of the route of transmission. Thus, whether an individual is infected with HIV intra-venously or intra-vaginally, the gut lamina propria is the first site that is depleted of the pivotal CD4⁺ T helper cells [2].

Although most pathogens enter humans through mucosal membranes, currently most vaccines are given through intra-muscular injections, which do protect hosts against pathogen-borne diseases. However, there is evidence to suggest that for long-term protection and in the absence

of pre-existing priming or immunity, mucosal vaccinations through oral, intra-nasal (IN), intra-rectal (IR), or intra-vaginal (IVAG) routes are more protective than systemic vaccinations [3, 4]. This is because it is expected that rapid local immune responses are required to inhibit or limit pathogen access to the interior of the host through mucosal surfaces. However, it remains a major challenge to design safe and effective mucosal vaccines despite the many advantages mucosal vaccines offer. Optimal strategies including type of antigens, delivery systems, and vaccination routes have yet to be determined [5]. So far, only half a dozen of the vaccines currently approved for human use are administered mucosally. Oral live attenuated polio vaccine, cholera vaccine, typhoid vaccine, rotavirus vaccine, and most recently the IN influenza vaccine are good examples of successful mucosal vaccines [6]. For HIV vaccination, despite considerable efforts in vaccine research and clinical trials, no effective vaccine is currently approved. It has been shown in some studies that parenteral immunization is not enough to achieve total protection from vaginally or rectally acquired HIV/SIV infection [7]. Mucosal immunization could be the best approach to achieve sustainable immune responses at mucosal sites of viral entry [7]. Mucosal HIV vaccines administered IN[8], IR [9,10], IVAG [11] and orally [12] have been shown to induce immune responses systemically and at mucosal sites of viral entry. Simultaneous immune responses at mucosal and systemic sites are induced by using appropriate adjuvants such as Cholera toxin, *Escherichia coli* heat-labile toxin, immunostimulatory CpG motifs, co-injection of cytokines and others. At present, there are no licensed immunopotentiating mucosal adjuvants or delivery systems for any vaccines. With increasing awareness that a mucosal vaccine holds the hope for protection against HIV [7], intense research is also being conducted to discover or design mucosal adjuvants and delivery systems.

In this review, previous and current approaches to design and develop vaccines that can protect against various routes of mucosal transmission of HIV will be discussed. Considering the ease and practical aspect of this vaccination route, the oral cavity could be an ideal route for HIV mucosal vaccination. While there oral HIV transmission rate through oral sex is 1–2%, vertical, mother to child transmission is as high as 12%. Nonetheless, there is a general lack of public awareness regarding the potential risk of acquiring HIV through oral transmission. Therefore, we will place special emphasis on the oral cavity, both as a portal of HIV entry and as a vaccination route to protect against various mucosal routes of HIV transmission.

2. Structure of the oral mucosa and oral transmission of HIV

The oral cavity has been known as a convenient route of drug and vaccine delivery for decades [13]. For example, the live attenuated (LA) polio vaccine was given as drops on the tongue of children, and vasodilators are frequently given to patients as sublingual pills or sprays, which are absorbed into the lingual veins in under a minute. More recently, sublingual vaccination has attracted more interest, particularly for vaccines against influenza [14]. However, this is not to be mistaken with oral immunization, which usually means passage of a vaccine through the harsh acidic compartment of the stomach, as well as the environment of the intestine with many potent degradative enzymes. Stimulation of mucosal immune responses by oral vaccination through the gastrointestinal tract has also been pursued vigorously, though relatively limited success has been achieved[15].

The oral cavity may be divided into the back region, which includes the pharynx and the Waldeyer's ring (tonsils) and the front region, which includes the sublingual and buccal (cheek) sub-regions [16]. The pharynx is further divided into three parts, the nasopharynx (posterior to the nose and superior to the soft palate), the oropharynx (posterior to the mouth) and the laryngopharynx (posterior to the larynx) [16]. The lymphoid tissue in the pharynx forms an incomplete circular lymphoid structure called the Waldeyer's ring. The lymphoid tissue is aggregated to form masses of lymph node (LN) called tonsils. The pharyngeal tonsil, known

as adenoids, is in the mucous membrane of the roof and posterior wall of the nasopharynx [16]. The palatine tonsils are LN at each side of the oropharynx between the palatine arches [16]. Unlike peripheral LN, which are not directly associated with the mucosal lumen, the surface epithelium of the tonsils, similar to the mucosa-associated lymphoid tissue (MALT) of the gastrointestinal tract (e.g. Peyer's patches), is in direct contact with the lumen. The palatine tonsils and adenoids are covered with lymphoepithelium consisting of ciliary and non-ciliary epithelial cells, goblet cells and microfold (M) cells, the latter showing many invaginating lymphoid cells [17]. Dendritic cells (DCs) are numerous within and underneath the epithelial layer of the tonsils and are in close contact with the neighbouring B and T cells [18]. Direct uptake of antigens through the epithelial cells of the tonsils has been demonstrated and suggests that the tonsils play a major role as local inductive sites for mucosal immunity [19].

The Waldeyer's ring, when compared to similar structures in rodents, is collectively referred to as nasal associated lymphoid tissue. Although the mouse model is often used in preclinical studies to predict the outcome of intra-nasal (IN) immunization in human, this remains to be proven [20]. Another organized lymphoid tissue, bronchus associated lymphoid tissue (BALT), has been demonstrated in rabbits, rats and guinea pigs, but rarely in humans, offering an explanation for lack of correlation between immune responses generated in these animals vs. in humans following IN or intra-tracheal (IT) immunizations [21]. Such differences may also play a role in the toxicity of adjuvants and delivery systems which are not evident in animals but are manifested more clearly in clinical trials [22].

The oral mucosa of the front region is covered by stratified squamous epithelium, which is about 50 cells thick in the buccal subregion, and somewhat thinner in the sublingual subregion [23]. The permeability of the sublingual mucosa is greater than the buccal which is greater than the palatal, based on the degree of keratinization of the tissues [23]. Epithelial penetration routes can be both transcellular (through cells) and paracellular (in between cells) [24]. Interestingly, contrary to the intestinal epithelia, the epithelial cells in the oral mucosa are devoid of tight junctions [24]. In contrast to the intestinal mucosa in which goblet cells secrete mucin, in the oral cavity, mucin is secreted by salivary glands as saliva [23]. The protein core of the salivary mucins consists of repetitive sequences that are rich in O-glycosylated serine and threonine residues, as well as many helix-breaking proline residues [25]. Up to 85% of the salivary mucins are composed of oligosaccharides [25].

The Langerhans cells of the buccal mucosa were found to be similar to those of the skin with regards to expression of the Major Histocompatibility Complex class II (MHCII), the mannose receptor DEC-205, CD11c and the lack of expression of the co-stimulatory molecules B7-1, B7-2, and CD40 [26]. More recently, it was shown in mice that three subsets of oral DCs exist, i.e. a minor subset of CD207(+) Langerhans cells located in the mucosa itself, a major subpopulation of CD11b(+)CD11c(-) and CD11b(+)CD11c(+) myeloid DCs at the mucosal/submucosal interface, and B220(+)120G8(+) plasmacytoid DCs in submucosal tissues [27]. Application of a hapten on the buccal mucosa induced recruitment of DCs and their expression of B7-2. Moreover, the hapten application induced contact sensitivity and induction of CD8+ and CD4+ T cells [26]. Furthermore, buccal immunization of mice with a measles virus nucleoprotein recruited DC and primed CD8+ CTL responses [27]. Sublingual allergen immunotherapy where the allergen is taken up via the R_cE receptor bound IgE also involves oral mucosal DCs [28]. These studies suggest that while buccal resident DC do not ordinarily express antigen presentation and activation markers, they can be induced to do so. Moreover, DC from other sites can be recruited to the buccal mucosa upon local infection.

In addition to the lymphoid aggregates in the epithelium, local draining lymph nodes also represent important inductive sites for local and systemic immunity following application of

antigens to the oral cavity. In the oral cavity, the lymphatic vessels of the parotid and submandibular glands drain to superficial and deep cervical LN [13]. Lymph from the end of the tongue drains to the superior deep cervical lymph node (LN), whereas lymph from the tip of the tongue drains to the submental LN. Lymph from the sides and the middle of the tongue drain to the inferior deep cervical LN and to the submandibular LN respectively [13]. Dimeric, multimeric and Secretory IgA, the most abundant immunoglobulin in external secretions, play critical roles in mucosal immunity through binding and carrying pathogens from the lamina propria or from within infected mucosal epithelial cells, as well as through hindrance of pathogen binding to apical surface of the mucosal epithelia [29]. Two subtypes of IgA exist in humans, IgA1 and IgA2. The latter is most frequent in the upper aerodigestive tracts, while the former predominates in the large intestine [29]. In this regard, tonsillar IgA+ cells are predominantly of the IgA1 subtype [30] providing further evidence that they function as local inductive sites that seed the mucosal effector sites of the upper aerodigestive tracts. In parotid saliva of HIV infected patients, IgA2 was found to be significantly increased, especially in those displaying oral manifestations of HIV-1 infection. In addition, IgA1 was shown to account for the majority of anti-HIV-1 antibodies [31].

The palatine tonsils and the adenoids comprise 30–35% CD3+ cells, 20–28% CD4+ cells, and 5–6% CD8+ cells and the majority of the T cells appear to express TCR $\gamma\delta$. While the activation marker IL2R (CD25) is upregulated on only 3–8% of the cells, CD28 is expressed on 23–36% of the cells and mostly in the adenoids [30]. T cells comprise about half of tonsillar intraepithelial mononuclear cells, with equal ratios of CD4+ and CD8+ cells. In the deeper inter-follicular regions the ratio of $\alpha\beta$ TCR+ to $\gamma\delta$ TCR+ cells is 10:1, whereas in the superficial areas a reduction in the number of $\alpha\beta$ TCR+ cells reduces this ratio to 2:1 [32]. Taken together, the presence of a lymphoepithelial structure, professional antigen presenting cells, e.g. DCs, FDCs [follicular dendritic cells] and the germinal centre machinery, as well as functional CD4+ and CD8+ T cells within the epithelial layers as well as deep in the LN suggest that the oro/nasopharyngeal lymphoid tissues act as both immune inductive and effector sites for the upper aerodigestive tract.

The percentage of HIV-positive saliva samples is 1–2% (33). This correlates well with the rate of oral HIV-transmission between homosexual men (34). Although HIV transmission through the oral mucosa is relatively a rare event [35], it can occur through genital-oral or breast-feeding routes. The oral-genital route of transmission applies to both the male and female genitals coming into contact with the oral cavity [35]. The first cases of oral HIV transmission were reported in the 1980's [36]. Clear demonstrations of oral transmission of SHIV or SIV in macaques have been reported since the 1990's [37]. Studies to investigate the mother to child vertical transmission of HIV were conducted in 1990's. One such study found that the vertical transmission rate was about 12.9% and while 83% of the infected children showed laboratory or clinical features of HIV infection by 6 months of age, by 12 months, 26% had AIDS and 17% died of HIV-related disease [38]. The presence of cell associated and cell free HIV in breast milk has been documented [39]. Macrophages were found to be the main source of infected cells in breast milk [40]. These data suggest that although HIV transmission through the oral mucosa appears to be a rare event, it can and does happen.

The presence of HIV virions in saliva, salivary glands, saliva-associated, buccal (cheek) epithelial cells is well documented [40,41]. However, there have been debates as to whether oral transmission, i.e. through saliva or oral fluids, is indeed a route of transmission. Secretory leukocyte protease inhibitor is a major human saliva protein shown to inhibit HIV [42]. Although the oral epithelia do not express HIV-binding receptors CD4, FC γ receptors for HIV-bound IgG, or MHCII, there are a number of Langerhans cells present within them, which potentially could take up HIV virions. Moreover, similar to the intestinal epithelia, the tonsillar epithelia express the glycosphingolipid galactosyl ceramide (GalCer), which is a documented

alternative receptor for HIV [43]. Moreover, salivary gland derived epithelial cell lines also expressed GalCer and CXCR4 [17]. CD4⁺ lymphocytes were readily detected within the oral/tonsillar mucosa [43]. Interestingly, human DCs also express GalCer [44]. HIV infection of human oral epithelial cells induced expression of beta defensins 2 and 3, which are well known small cationic molecules active at mucosal membranes [45]. Salivary mucins, MUC5B and MUC7, were shown to significantly inhibit HIV growth [46]. Non-traumatic oral exposure of SIV resulted in detection of SIV RNA in oral and esophageal mucosa, tonsils and tissues proximal to stomach by 1 day post exposure and in regional and peripheral lymph nodes by 2 days post exposure [47]. Expression of innate immune response genes (type I and II interferons, CXCL9 and CXCL10) in the oral mucosa vs. the tonsils correlated with slower disease progression, following oral SIV exposure [48]. Decreased salivary secretory IgA (SIgA) has been correlated with HIV infection and SIgA has been shown to neutralize multiple HIV strains [49]. Thus, many innate and adaptive defense mechanisms strongly limit oral HIV transmission.

Breast-feeding by HIV-infected mothers is a well-established route of HIV transmission [50]. Moreover, mucosal exposure of infants correlates with maternal plasma virus levels [51]. In the SIV/macaque model, it was demonstrated that increased diversity of SIV envelope variants was associated with improved survival of pre-vaccinated infant macaques [52]. It is believed that although oral transmission is less common than genital transmission, it appears multiple exposures per day for many months eventually result in transmission and infection of the child.

3. Oral vaccination strategies against oral and other mucosal HIV transmission routes

The oral vaccination route, i.e. passage of the vaccine through the stomach and the intestine, has potential as a delivery site for prospective HIV vaccines that may induce both systemic and mucosal immune responses. During early 1990's, several oral live attenuated *Salmonella* vector HIV-1 vaccines were shown to elicit both mucosal and systemic immune responses [53]. More recently, oral vaccination with a recombinant *Salmonella* vaccine vector evoked systemic HIV-1 subtype C Gag-specific CD4⁺ Th1 and Th2 cell immune responses in mice [54]. Oral immunizations with recombinant *Listeria monosytogenes* expressing HIV-gag controlled viral loads following vaginal challenge with feline immunodeficiency virus (FIV), and their intestinal CD4⁺ T cells were preserved [55]. A similar vaccine tested in rhesus monkeys showed that Oral priming/oral boosting induced Gag-specific cellular immune responses, whereas oral priming/intra-muscular boosting induced systemic as well as mucosal anti-Gag antibodies [56]. An oral recombinant *Mycobacterium bovis* bacillus Calmette-Guérin expressing HIV-1 antigens as a freeze-dried vaccine induced long-term, HIV-specific mucosal and systemic immunity [57]. In rhesus macaques, oral administration of enteric coated capsules containing an adenoviral vector encoding HIVgag, induced mucosal (salivary and vaginal) and systemic (serum) antibody responses as well as systemic and intestinal T cell responses [58]. No mention of rectal antibody or T cell responses was made in this report. Moreover, oral and nasal priming with a replication-competent adenovirus-based vector expressing SIV antigens followed by boosting with a protein-based SIV antigen induced peripheral blood immune responses and protected against rectal challenge with homologous (vaccine strain) rectal challenge [59]. However, IN priming with a similar adenovirus-based vector combined with protein boosting, failed to protect against intra-venous SHIV challenge [60]. As discussed under the Expert Opinion section, the adenovirus-based vector failed in a major clinical trial.

Oral administration of poly(dl-lactide-co-glycolide)-encapsulated plasmid DNA encoding HIV gp160 in mice induced both systemic and mucosal immune responses while intramuscular injection only induced systemic immune responses [61]. An orally administered therapeutic HIV vaccine using pooled, inactivated HIV antigens showed compelling clinical responses in

early-stage patients in Thailand [62]. A virus like particle (VLP) vaccine encoding HIV gp120 induced both systemic and mucosal immune responses when administered orally [63]. Edible HIV vaccines based on the HIV tat gene expressed in plants, such as tobacco [64], tomato [65], or spinach [66] also showed immune responses in mice. However, protection in primate models of AIDS using these approaches have not been reported.

It is important to note that oral or nasal administration of non-replicating protein-based antigens in the absence of immune-enhancing adjuvants and delivery systems generally does not induce immunity but rather systemic unresponsiveness upon systemic administration of the same antigen. This process is termed mucosal tolerance. The role of mucosal CD4⁺CD25⁺ and CD4⁺CD25⁻ regulatory T cells (Treg), that secrete TGF β , IL-4, and IL-10, in mucosal tolerance induction for vaccine development against infectious [67] vs. autoimmune diseases [68] is currently under intense research.

These reports demonstrate a variety of approaches and technologies for design of oral vaccination against HIV transmission. However, the feasibility of these approaches, specially their safety profile, in humans remains to be established.

4. Structure of the vaginal mucosa and vaginal transmission of HIV

Most HIV infections occur via heterosexual transmission through the female reproductive tract. The innate immune system, the first line of immunological defense against vaginally transmitted pathogens, may exert considerable influence in the vaginal response against HIV [69]. CC-chemokines, which would block the CCR5 coreceptor necessary for binding and infection with HIV, were suggested to be increased in protected animals or humans [70]. However, a recent study did not find evidence of an increase of such chemokines at 1–3 days after challenge with SHIV, in vaccinated/protected rhesus macaques [71].

The vaginal mucosa is covered with multi-layered squamous epithelia, while the uterus, cervix and fallopian tubes are covered with pseudo-squamous and simple columnar epithelia. Underneath the epithelial layers of the vagina, uterus and fallopian tubes is the lamina propria compartment comprising a large array of B cells, CD4⁺ and CD8⁺ T cells, and antigen-presenting cells (APCs) [72]. The presence of lymphoid aggregates in the female genital tract has also been reported, although whether these aggregates have follicle associated epithelium, as is the case with nasal-associated lymphoid tissue (NALT) and Peyer's patches, remains to be elucidated [72]. DCs and CD8⁺ T with cytotoxic activity cells are found interspersed within the squamous epithelium of the vagina [73]. Thus, the vaginal mucosa contains DCs as well as CTL and can mount anti-viral cytotoxic T cell responses that can be protective.

HIV target cells are found in high numbers in the ectocervix and vagina as well as in the endocervix and uterus. Susceptibility to infection may vary during the menstrual cycle, because of the hormonal regulation of innate and adaptive immune responses [74].

In the rhesus macaque/SIV model, it was shown that DC interspersed in the vaginal epithelium, take up viral particles from the vaginal lumen, become persistently infected, and delivered the virions to the draining internal iliac lymph nodes [75–78]. Alternatively, it is possible that cell-free or cell-associated HIV may reach the simple columnar epithelia of the cervix and penetrate the mucosa via transcytosis to infect lamina propria target cells such as CD4⁺ T cells [37,51,79]. The cervicovaginal mucosa also contains B cells. The major portion of antibodies from the cervicovaginal mucosa is of the IgG isotype, although lower levels of IgA are also found. The presence and concentration of antibodies in the female genital tract is subject to variations based on the hormonal cycles [80].

Male to female and female to male HIV transmissions do occur and in this context efforts have been made to understand the cellular structure and the transmission mechanisms of the male genital tract. Similar to the female genital tract the male genital tract (MGT) is also a major HIV target and transmission route. Interestingly, the odds of male-to-female transmission were found to be significantly greater than female-to-male transmission[81]. It has been shown that several organs of the MGT, including testes, epididymides, prostate and seminal vesicles can become infected by HIV/simian immunodeficiency virus (SIV) and likely to contribute to semen viral load during the early and chronic stages of the infection[82,83]. Similar to the FGT fluids, seminal plasma contains mostly IgG, and relatively low levels of IgM, and IgA. This IgA is found in the form of secretory IgA, polymeric IgA, and monomeric IgA. Whereas in FGT fluids the ratio of IgA2 to IgA1 is slightly greater in seminal plasma, similar to blood plasma, IgA1 predominates. Moreover, the IgG subclasses in seminal fluid also resemble that of serum[84].

5. Vaccination strategies against vaginal HIV transmission

Considerable efforts have been undertaken to develop preventive vaccines against HIV transmission through vaginal or genital mucosa by IVAG, IN, oral, or systemic routes of vaccination. A fusion protein consisting of cholera toxin B subunit (CTB) and HIV gp41 peptide induced systemic and vaginal antibody responses following IN priming and systemic boosting immunization of mice, only in the presence of cholera toxin (CT) as a mucosal adjuvant [85]. In another study, CT was also efficient for induction of anti-C4/V3 (HIV-envelope) peptides, but only after 4 doses [86]. Because of the toxic effects of CT in humans, the use of this adjuvant in a licensed clinical product is not possible. Also, IN, intra-peritoneal (IP), but not oral, vaccinations with VLP derived from clade A HIV-1 gp120 induced vaginal and intestinal neutralizing antibody responses against homologous and heterologous virus strains [87]. These studies provided evidence that vaccinations with inert protein-based vaccines could induce immune responses against the HIV envelope gp120 glycoprotein. Because neutralizing antibodies against gp120 are generally accepted to be important in HIV vaccine development, these data were encouraging, even though the use of a toxin-based adjuvant precluded such vaccines for human use.

Efforts on HIV vaccine development include also plasmid DNA vaccinations. Plasmid DNA adsorbed to cationic microparticles, as opposed to plasmid DNA alone, induced local and systemic immune responses against HIV-gag following IN immunizations. IN immunizations with a cationic lipid/DNA adjuvant, N3, followed by IN immunizations with HIV-gp160 peptide with an anionic L3 adjuvant, induced broad antibody responses against HIV-1 subtype A, B and C [88]. In nonhuman primates, IM DNA priming followed by IN fowl pox boosting induced limited protection in acute phase of infection against homologous SHIV challenge [89]. IN priming with DNA encoding HIV envelope gp160 and boosting with HIV envelope gp41 peptide induced long term serum IgG responses [90]. A plasmid DNA vaccine encoding HIV-2 tat, nef and gag in the presence or absence of granulocyte-macrophage colony stimulating factor and B7-2 costimulatory molecule, was administered to baboons, which were then challenged IVAG. This vaccine, with or without the adjuvants, induced reduced viral loads following vaginal challenge with HIV-2 [91]. While these studies provided evidence that plasmid DNA vaccinations can induce immune responses in small animal models, in general, plasmid DNA vaccinations induce poor responses in primates, including non-humans and humans, and prime/boost strategies with live attenuated viruses or bacteria have been required to induce detectable immune responses in primates.

IN and IVAG routes of vaccination against HIV have been tested. Staats et al [92] demonstrated that IN administration of a HIV-1 peptide vaccine (T1Sp10 MN) with CTB in mice induced significant levels of anti-peptide serum IgG titers and anti-HIV-1MN neutralizing antibody

responses. Vaginal anti-peptide IgG and IgA titers were also induced. In this study vaginal HIV-1 IgA was associated with secretory component, suggesting transepithelial transport of IgA into vaginal secretion. In addition, IN immunization with HIV gp120 in an emulsion adjuvant also induced antibodies in the vaginal washes of mice, although it was not clear whether these antibodies were produced locally or were serum transudates [93]. Although there is no evidence to indicate the presence of M cells in the vaginal mucosa [94], intra-vaginal (IVAG) immunization in humans induced local antibody responses [95]. However, IVAG immunization protocols in small animal models have not generally met with great success, despite the use of novel delivery systems and adjuvants [96]. More recent reports though, showed that vaginal immunizations were better than IN or intra-muscular immunizations with alpha virus based replicon particles encoding HIV-1 gag protected against IVAG challenge with vaccinia virus encoding HIV-1 gag [97,98]. Moreover, the local immune response in the vagina is subject to significant hormonal regulation, with major changes in local antibodies at different stages of the menstrual cycle [99]. A study in mice showed that the IN route of immunization was more effective than the IVAG route for the induction of immune responses in the vagina [100]. In female humans the IN route of immunization may be exploited for the induction of genital tract antibody response [101].

IN priming with HIV-env-expressing influenza virus and IN boosting with HIV-env-expressing vaccinia virus (VV) in mice, induced systemic cellular responses in spleen and local responses in the genitoretal draining lymph nodes [102]. IN priming with DNA and IN boosting with VV expressing HIV-env induced mucosal and systemic humoral and cellular responses [103]. IN priming with a replicating Tiantan VV followed by IM boosting with DNA, both expressing HIV-gag resulted in vaginal IgA, serum IgG and systemic T cell responses [104]. Interestingly, previously, the reverse of this prime/boost strategy using DNA for priming and Tiantan VV for boosting had demonstrated induction of IgA and cytokine responses in lung and genital tracts of mice [105].

Combination of parenteral and mucosal immunizations with live attenuated recombinant vesicular stomatitis virus (rVSV) was shown to prevent AIDS in rhesus macaques [106]. A comparison of IN and IM routes of immunization with rVSV in rhesus macaques demonstrated that the IN route induced higher cellular as well as nasal and saliva responses, but both routes of immunization conferred protection against vaginal SHIV challenge [107]. Recombinant Influenza A virus containing an HIV-nef insert, that did not replicate in the respiratory tract, was used for a single IN immunization of mice and induced CD8⁺ T cell responses in spleen, lymph nodes draining the respiratory tract and urogenital tract [108]. Moreover, a chimeric influenza virus, that did replicate in the respiratory tract of mice, containing an HIV-env neutralizing epitope, induced humoral responses in spleen, lungs and urogenital tracts [109]. Interestingly, IN immunization with inactivated influenza virus enhanced immune responses to co-administered SHIV VLP, suggesting that influenza virus viability may not be required for the adjuvant action of influenza viruses [110]. However, while HIV-gp120 antigen formulated in PR8-influenza-ISCOM adjuvant induced systemic cytokine and antibody responses in rhesus macaques following the targeted lymph node immunization, no responses were detected following IN immunizations, even though IN immunizations induced immune responses in mice using the same antigen/adjuvant/delivery systems [111]. This finding underscores the importance of the species specific differences in designing vaccines.

Inactivated HIV or SHIV-capturing nanoparticles have been produced and used to induce vaginal antibody responses in mice and rhesus macaques following IN immunizations [112]. Moreover, inactivated HIV-1 plus CpG adjuvant induced genital CTL and antibody responses in mice that were subsequently protected against vaginal challenge with recombinant VV [113]. There have also been examples of IN immunizations with recombinant BCG bacteria encoding an HIV-1 antigen [114] or heat-inactivated bacteria conjugated to HIV-env antigen

[115]. Reports on these approaches in non-human primates are scarce and thus their viability as an effective anti-HIV-1 vaccine for human use remains to be explored. As stated above, LA or inactivated HIV-1 viruses will most likely not serve as a vaccine candidate for human use due to serious safety concerns. Intravaginal vaccination (priming) with attenuated, recombinant *L. monocytogenes*-gag (rLm-gag) followed by a boost with replication-defective rAd5 (adenovirus serotype 5)-gag induced strong cellular immunity in the vaginal tissues and protection against vaginal challenge [116].

A phase I clinical study in France employing a recombinant protein HIV-1 gp160MN/LAI with or without DC-Chol adjuvant (a cationic lipid 3 β -(*N*-(*N'*, *N'*-dimethylaminoethane) carbamoyl) cholesterol) administered by the nasal or vaginal route showed that the mucosal HIV vaccine was well tolerated when administered by the nasal or vaginal route, but no anti-gp160 antibody activity was found between week 4 and week 48 in serum, saliva, or cervicovaginal and nasal secretions [117].

Thus, although the vaginal mucosa contains the necessary immunological machinery to mount a local immune response, the IN immunization appears to be a more suitable route. However, it remains to be seen whether in the resting memory phase a more rapid local response is induced in the vaginal mucosa following intra-vaginal immunization compared to IN immunization.

6. Structure of the rectal mucosa and rectal HIV transmission

The rectal mucosa is also a major mucosal HIV transmission route. The transmission can be through homosexual or heterosexual anal intercourse [118]. Sexually transmitted diseases, such as Chlamydia, syphilis, and gonorrhea can often facilitate rectal HIV transmission [119, 120]. The rectal mucosa of several mammalian species, including humans, contains macroscopically invisible solitary lymphoid nodules that resemble Peyer's patches of the small intestine in their cellular structure and phenotype. These structures are overlaid with microfold (M) cells that are specialized in antigen uptake. Of note, both the rectal and vaginal mucosa are drained by the iliac lymph nodes and there is indirect evidence that SIgA-secreting cells in the vaginal mucosa originate from the solitary lymphoid nodules of rectum [118]. In non-human primates as well as in humans [121], the rectal and small intestinal lamina propria contain high numbers of CD69+ macrophages that are concentrated under the single layer of epithelial cells (enterocytes) whereas cells with dendrites, that are far fewer in number (most likely DC), form a reticular frame work throughout the lamina propria. Thus, the rectal mucosa contains many HIV targets as well as CTL and plasma cells in both the diffuse lamina propria and the well-organized lymphoid nodules.

7. Vaccination strategies against HIV rectal transmission

The rectal mucosa may serve as a vaccine delivery route and because the vaccine does not have to go through the entire digestive tract and the intestine, lower amounts of antigen are required for intra-rectal compared to oral immunization. Much efforts have been focused on HIV DNA vaccines administered IR. IR immunization with a replication-deficient recombinant VV expressing HIV 89.6 env protein induced both mucosal and systemic CTL responses in mice [122]. Rectal vaccination with a HIV-1 gp120 DNA vaccine delivered with an attenuated *Salmonella* recombinant strain induced higher immune responses than oral vaccination in mice [123]. A simian-human immunodeficiency virus (SHIV) DNA vaccine applied IR to macaques also provided systemic and mucosal protection against AIDS [124]. A DNA prime/VV boost immunization containing multiple HIV genes controlled viral loads following intra-rectal challenge [125]. In a non-human primate model, IN, intra-muscular and IR immunizations with a live attenuated pox virus (NYVAC) expressing an immunodominant CD8+ CTL gag-epitope, induced CTL responses in the small intestine [126].

Although rectal vaccination for HIV may induce both systemic and mucosal protections, it may not be an attractive route of immunization for socio-ethical reasons. While IR vaccination may be necessary for protection against rectal HIV transmission, systemic or oral vaccination may induce effective rectal immune responses too. In support of such a notion, oral/tonsillar application of an inactivated simian immunodeficiency virus (SIV) mac239 induced partial protection against rectal challenge with SIVmac239 in rhesus macaques [127].

8. Expert opinion

Any investigator who attempts to design an HIV vaccine soon realizes that, unlike e.g. polio and influenza, the immune responses that correlate with protection against HIV are not well established. Therefore, there is general agreement that at least both broad neutralizing antibodies and CTL responses may be required. From a mucosal vaccinologist's perspective, however, such responses are needed both at the mucosal portals of entry as well as systemically. HIV is transmitted through three different mucosal routes, namely the oral, the rectal and the vaginal mucosa. An early clinical study showed that local vaccination either IVAG or IR induced the highest immune responses at the vaccination sites [128]. However, it is still an open question as to whether local vaccination is superior to systemic or distant mucosal vaccination for protection against infection or disease in clinical settings. Based on observations on how HIV and simian immunodeficiency virus (SIV) are transmitted mucosally and spread systemically, a multi-level barrier model that would confer generalized immunity against HIV transmission has been proposed [129,130]. In exposure to the genital and rectal mucosa, the first immune barrier against HIV is locally produced SIgA and IgG in the vaginal and rectal lumen. If the virus load is too high and some virions pass through the epithelial barrier, and reach the lamina propria, a second immune barrier in the form of neutralizing antibody-secreting cells as well as CTL effector cells is required. If the immune responses generated at the lamina propria, the second barrier, is not sufficient, the virion or virus infected cells will reach the third barrier, the draining iliac lymph nodes via the lymphatic system. Here, there will be a need of neutralizing antibodies as well as effector CTL. Finally, to prevent systemic spread and disease, a fourth, systemic, immune barrier, will be needed, again in the form of both neutralizing antibodies and CTL. It is important to note that due to the difficulties and the labor intensive and time-consuming nature of isolation of mucosal lamina propria cells and time, most studies have focused on measuring systemic responses even in murine and non-human primate models. It is also clear that isolation of lamina propria cells from pinch biopsies from the rectal or cervicovaginal mucosa of human subjects HIV-infected individuals as well as uninfected individuals at high risk for HIV infection yields relatively few cells and involves addressing serious ethical and regulatory issues before vaccine trials are permitted. Hence, the non-human primate models of SIV and SHIV infection have been used to elucidate the role of mucosal and systemic antibodies and T cell responses as correlates of protection, even though it has become increasing evident that such models may ultimately not accurately mirror the protective immune responses in humans.

Vaccination against oral transmission is generally a new field and requires more in depth studies. Oral vaccination strategies against HIV have mostly targeted the small intestine and investigators have not addressed whether their oral vaccines could actually protect the oral mucosa. Increased awareness of the oral mucosa as a potentially important route of transmission in infants of HIV-infected mothers may promote more expanded research in this area.

The rectal route of transmission is important not only in homosexual but is becoming increasing evident as an important route of transmission among heterosexuals who engage in anal sex. While there is some preliminary evidence that oral immunization may protect against rectal HIV transmission, general vaccination studies suggest that rectal immunization may prove

more protective. To date, relatively few studies have compared rectal vs. oral vaccination and protection against rectal challenge.

The vaginal route of transmission has attracted the most focus because worldwide it is the most prevalent route of mucosal transmission. IN immunizations has been the most popular route of vaccination against vaginal transmission of HIV. It is currently not known why IN immunizations induce immune responses in the vaginal mucosa, which may also be protective. The most important obstacle in mucosal immunizations, including those against HIV, is the design and development of safe and effective immunopotentiating adjuvants and delivery systems. The use of live attenuated viral and bacterial delivery systems would most likely diminish the requirement of immunopotentiating adjuvants. However, live attenuated (LA) delivery systems have safety as well as anti-vector pre-existing immunity issues. On the other hand, while DNA- and protein-based vaccines are generally safer than LA delivery systems, they require the use of immunopotentiating adjuvants. There is currently intense search for finding safe and effective immunopotentiating adjuvants, particularly those that can induce a TH1-type immune response, or at least do not cause an overt TH2-type immune response [131]. This is because a TH2-inducing adjuvant given IN could result in adverse mucus production, vasoconstriction and asthma-like symptoms. Whether there will be a vaccine that can protect against transmission through all three routes that have been discussed here, is subject of future research endeavors. As of now, almost all investigators focus on only one mucosal route of transmission. Design of a vaccine that can protect any of these mucosal routes against transmission will be a great achievement in itself and will also pave the way for protection against transmission through the other routes.

The future of HIV vaccine research could see a surge in interest in early events following mucosal transmission. This will include studying both the cells that are initially infected as well as the downstream intra-cellular and inter-cellular events. Particular focus will be placed on innate immune responses at the mucosal portals of entry and how these are or can be linked to adaptive immune responses including memory type responses. The majority of vaccine studies involving HIV or any other pathogen focus on acute responses and relatively few studies deal with long-term protection and the generation and maintenance of memory responses. Because an effective HIV vaccine should protect the host beyond the acute phase of the immune response following vaccination, more emphasis should be placed on whether and how a vaccine can induce long-term protection.

Recently, two major HIV vaccine trials in which volunteers were vaccinated intra-muscularly failed to show efficacy. Indeed, the Merck vaccine trials that used a live attenuated adenovirus vector appeared to increase the risk of infection among vaccinated volunteers. More recently, using a pox virus priming and gp120 protein boosting strategy, a clinical HIV vaccine trial supported by Sanofi-Pasteur showed limited (30%) efficacy [132]. Because live attenuated vectors administered through mucosal or systemic routes potentially migrate to distant mucosal tissues, they may have a greater potential to protect against mucosal HIV transmission. However, safety issues may remain a major hurdle for use of any live attenuated vectors as preventive vaccine delivery systems.

The interdependence of lymphocyte trafficking on the routes of vaccination or infection is noteworthy. Several studies have shown a role for the $\beta 7$ integrins $\alpha 4\beta 7$ and $\alpha E\beta 7$, which both bind to the mucosal vascular addressin MADCAM-1 and E-cadherin respectively, in homing of lymphocytes to the gastrointestinal and genitourinary tracts [133,134]. While the role of $\alpha 4\beta 7$ expression by antigen-specific B cells in the gastrointestinal tract following oral immunizations or challenge is well established [135], its role on antigen-specific B cells in the respiratory or genitourinary tract following IN or combinations of IN and systemic immunizations is less clear, as roles for both $\alpha 4\beta 7$ and L-selectin have been suggested [136,

137]. Although several studies have reported the characterization of CD4⁺ T cells in the murine female genital mucosa, reports on the characterization of CD8⁺ T cells at this site are more limited [138,139,140,141]. $\alpha 4\beta 7$ has been implicated in CD8⁺ T cell homing to the intestinal mucosa in protection against rotavirus [142], and CD8⁺ cells have been shown to increase in size in the vagina following infection with herpes simplex virus type 2 (HSV-2), particularly after immunization [142]. Chemokines such as RANTES which interact with the chemokine receptor CCR5, an important HIV coreceptor, have been shown to induce an influx of cells to sites of infection [143,144].

The importance of homing receptors and cell trafficking in HIV infection was recently demonstrated in that adenovirus vector vaccination induced expansion of memory CD4 T cells with a mucosal homing phenotype, i.e. $\alpha 4\beta 7$ + and CCR9+, as well as increased CCR5 expression that were susceptible to HIV-1 [145]. Interestingly, HIV-1 infection was found to be associated with a significant increase in mucosal but not peripheral $\gamma\delta$ + T-cell populations [146]. These and similar reports underscore the need to study the role for resident mucosal cells as well as peripheral cells that acquire mucosal homing receptors in transmission as well as disease progression following HIV infection regardless of the route of transmission. This raises a fundamental question in mucosal vaccinology that also pertains to HIV vaccines, i.e. are mucosal vaccinations required to prevent infection or disease? In the rhesus macaque model of SHIV challenge, it has been shown that intra-muscular vaccinations with live, replicating and non-replicating vaccine delivery systems and adjuvants resulted in lack of plasma viral RNA and/or preservation of CD4+ T cells in peripheral blood. The problem with such studies is that the IR or IVAG mucosal challenge was performed within weeks of the final vaccination at a time when serum transudates were prevalent at the mucosal site of transmission and hence the role of locally derived antibodies in a memory setting, i.e. a long time after vaccination, remains obscure. Moreover, most such studies do not address the issue of whether such systemic vaccinations also preserve mucosal CD4+ T cells that are the major targets of HIV and whether HIV or SIV or SHIV is still present at the site of the mucosal challenge at various times after challenge. These issues are of utmost importance as it is well-established that HIV, SIV or SHIV can remain dormant in the mucosa and later cause disease.

Article Highlight Box

- While HIV transmission through the oral mucosa is rare, it does occur and yet the oral route of HIV transmission is a relatively neglected research area.
- The structure and cells of the oral mucosa, epithelia and the tonsils suggest that many HIV targets as well as potential immune effector functions exist at this site.
- There is considerable difference between sublingual vaccination, i.e. uptake through the oral and tonsillar mucosa, and oral vaccination, i.e. uptake through the small intestinal mucosa.
- While relatively few studies have addressed the possibility of oral or sublingual vaccination to protect oral HIV transmission, most studies have focused on oral vaccination strategies to prevent vaginal and rectal HIV transmission.
- The very first HIV targets in the vaginal and rectal mucosa and the early downstream immunological events are deduced from rhesus macaque and SIV studies.
- The cells and structure of the vaginal and rectal mucosa contain an abundance of HIV targets, but also many effector B cells and CTL, and, as yet less well-defined, innate immune responses.
- Many vaccination strategies through the oral, intra-nasal, and intra-vaginal routes against vaginal and rectal HIV transmission have been employed.

- Effective and, equally important, safe mucosal vaccine adjuvants and delivery systems are required to design a mucosal HIV vaccine.

Acknowledgments

Declaration of interest

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