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Intranasal delivery of vaccines against HIV

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HIV poses a serious health threat in the world. Mucosal transmission of HIV through the genitourinary tract may be the most important route of transmission. Intranasal immunisations induce vaginal and systemic immune responses. Various protein-, DNA- and RNA-based immunopotentiating adjuvants/delivery systems and live bacterial and viral vectors are available for intranasal immunisations, and these systems may differ in their ability to induce a specific type of immune response (e.g., a cytotoxic T cell versus an antibody response). As the protection against HIV may require both cytotoxic T cell and antibodies, a combination of adjuvants/delivery systems for combinations of mucosal and parenteral immunisations may be required in order to develop a protective anti-HIV vaccine.

Keywords: adjuvants, delivery systems, HIV, intranasal immunisation, mucosa, vaccine

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1. Introduction

Currently, HIV has infected 40 million people around the globe, and each day ~ 15,000 additional infections occur. There is a general consensus that the most effective way to prevent new infections or disease is to introduce a prophylactic vaccine. However, over two decades of attempts to develop an anti-HIV vaccine have resulted in no licensed vaccine for human use. One of the major issues contributing to the difficulty of developing a protective anti-HIV vaccine is the lack of a consensus on the correlates of protection. In protected non-human primates challenged with simian immunodeficiency virus (SIV) or simian-human immunodeficiency virus (SHIV), or in exposed, uninfected humans, either cytotoxic T cell (CTL) or neutralising antibodies can exclusively correlate with protection. However, there is a general consensus that both CTL and neutralising antibodies are important to mediate protection. Neutralising antibodies need to be broadly reactive to neutralise multiple primary isolates. There is increasing agreement that the CTL and neutralising antibodies should be present at both mucosal sites of HIV entry, as well as in the draining lymph nodes (LNs), and systemically.

Of the several mucosal routes available for mucosal immunisation (i.e., oral, nasal, vaginal and rectal), the nasal route is the most popular for human vaccinations for a number of reasons. The nostrils are readily exposed and available for administration of vaccines by health professionals or even by self-administration. Thus, in terms of ease of administration, the intranasal (IN) route resembles the oral route. However, although the oral route has been used for drug delivery for centuries and is much preferred, IN immunisation generally requires much lower doses of antigen, with important implications for many, often costly, recombinant antigens. Lower doses are possible via the IN route mainly because IN immunisation, as opposed to oral or intrarectal (IR) immunisation, for example, does not expose antigens to low pH and proteases. An important benefit of IN immunisation, in comparison to oral immunisation, is that IN immunisation has been shown to induce potent responses both in the upper and lower respiratory and the genital tracts, through as yet undefined mechanisms [1-3]. Indeed, it seems that in contrast to local immunisation of

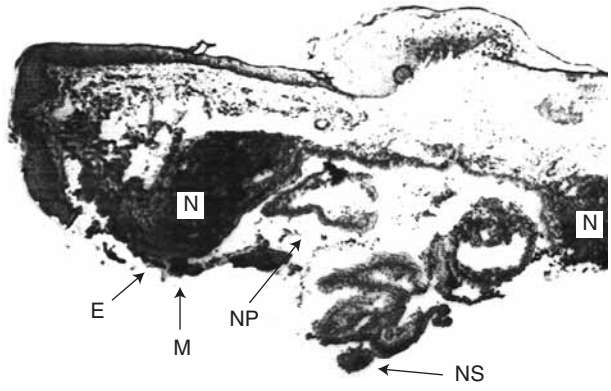

Figure 1. Haematoxylin staining of a 7-µm frozen, acetone-fixed, section of nasal-associated lymphoid tissue from a mouse.

E: Epithelial cell; M: M cell; N: Nasal-associated lymphoid tissue; NP: Nasal passage; NS: Nasal septum.

the genital tract, IN immunisation induces vaginal and systemic responses that in some cases are more potent compared with that induced by vaginal immunisations [4-9]. Thus, compared with rectal or vaginal immunisations, the IN route is more readily accessible, culturally more acceptable, and induces better mucosal and systemic immune responses.

2. Cellular components of the upper and lower respiratory and the genitourinary tracts

To understand how delivery of a given drug or vaccine results in its uptake, it is imperative to form an idea of the cellular structure of the site of delivery and how antigen or drug uptake occurs. The uptake of any vaccine at mucosal surfaces occurs by or through the epithelial layer, followed by uptake and presentation by antigen-presenting cells (APCs).

In primates, the palatine, lingual and nasopharyngeal tonsils form the Waldeyer's ring. The counterparts of the Waldeyer's ring in rodents are similar but not identical structures, collectively referred to as nasal-associated lymphoid tissue (NALT; Figure 1). Another organised lymphoid tissue, bronchus-associated lymphoid tissue, has been demonstrated in rabbits, rats and guinea-pigs but rarely in humans. This can offer an explanation for the lack of correlation between immune responses generated in these animals versus in humans following IN or intratracheal immunisations. Waldeyer's ring is strategically placed for sampling at the portals of both gastrointestinal and respiratory tracts [10].

The structure and localisation of the nasopharyngeal lymphoid tissues and accumulating data suggest that they function as local inductive sites for the upper aerodigestive tract. The pharynx is 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) [11]. The lymphoid tissue in the pharynx forms the incomplete circular lymphoid structure of Waldeyer's ring. This lymphoid tissue is aggregated to form the tonsils. The pharyngeal tonsil, known as the adenoids, is in the mucous membrane of the roof and posterior wall of the nasopharynx [11]. The palatine tonsils are the LNs that are at each side of the oropharynx between the palatine arches [11]. Unlike peripheral LNs, which are not directly associated with the mucosal lumen, the surface epithelium of the tonsils, similar to the mucosal-associated lymphoid tissue of the gastrointestinal tract (e.g., Peyer's patches), is in direct contact with the lumen. The adenoids are covered with lymphoepithelium consisting of ciliary and non-ciliary epithelial cells, goblet cells and M cells; the latter showing many invaginating lymphoid cells [12]. 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 [13]. 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.

Two subtypes of IgA exist in humans, IgA1 and IgA2. The latter is most frequent in the upper aerodigestive tracts, whereas the former predominates in the large intestine [14]. In this regard, tonsilar IgA+ cells are predominantly of the IgA1 subtype [15,16] providing further evidence that they function as local inductive sites that seed the mucosal effector sites of the upper aerodigestive tracts. The palatine tonsils and the adenoids comprise 30 – 35% CD3+ cells, 20 – 28% CD4+ cells and 5 – 6% CD8+ cells, and 25 – 35% express TCR$\alpha\beta$, with only 2 – 3% expressing TCR$\gamma\delta$. Although the activation marker IL-2R (CD25) is upregulated on only 3 – 8% of the cells, CD28 is expressed on 23 – 36% of the cells, and mostly in the adenoids [16]. T cells comprise ~ 50% of tonsilar 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 [17]. Taken together, the presence of a lymphoepithelial structure, professional APCs (e.g., DCs, follicular DCs and the germinal centre machinery) as well as functional CD4+ and CD8+ T cells within the epithelial layers and deep in the LNs suggest that the oro/nasopharyngeal lymphoid tissues act as both immune inductive and effector sites for the upper aerodigestive tract (Figure 2).

The vaginal mucosa is covered with multi-layered squamous epithelia, whereas 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 APCs [18]. 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 NALT and Peyer's patches, remains to be elucidated [18,19]. DCs and CD8+ T cells with cytotoxic activity are found interspersed within the squamous epithelium of the vagina [20-22]. Thus, the vaginal mucosa contains DCs as well

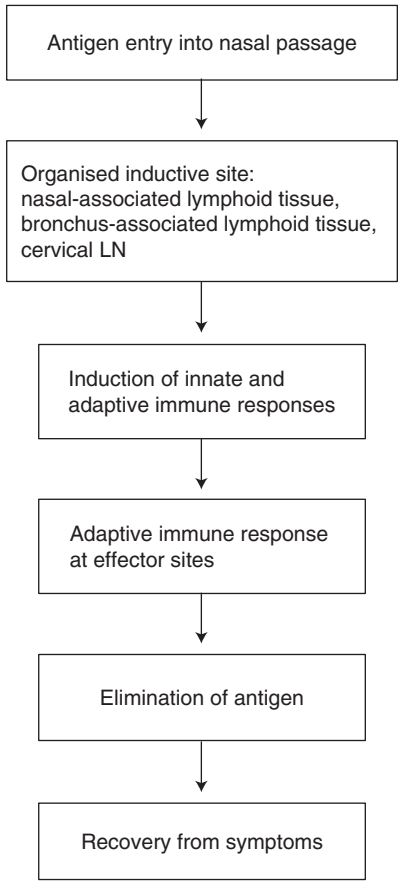

Figure 2. Sequence and location of immunological events following intranasal immunisation.

as CTLs, and can mount antiviral cytotoxic T cell responses that can be protective. The vagina is considered to be a component of the common mucosal immune system, and oral immunisation in mice with microparticles has been shown to induce a vaginal antibody response [23]. In addition, IN immunisation with microparticles also induced antibodies in the lower genital tract of mice [24]. Although there is no evidence to indicate the presence of lymphoid follicles or M cells in the vaginal mucosa [25], intravaginal immunisation in humans induced local antibody responses [7]. However, intravaginal immunisation protocols in small animal models have generally not been met with great success, despite the use of novel delivery systems and adjuvants [26-28]. However, more recent reports showed that vaginal immunisations were better than IN or intramuscular (IM) immunisations with alphavirus-based replicon particles encoding HIV-1 gag protected against intravaginal challenge with vaccinia virus (VV) encoding HIV-1 gag [29,30]. 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 [31]. A study in mice showed that the IN route of immunisation was more effective than the intravaginal route for the induction of immune responses in the vagina [8]. In female humans the IN route of immunisation may be exploited for the induction of genital tract antibody response [9]. Thus, although the vaginal mucosa and its putative inductive sites contain the necessary immunological machinery to mount a local immune response, the IN immunisation seems 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 intravaginal immunisation compared with IN immunisation.

3. Intranasal delivery of recombinant protein- and peptide-based HIV vaccines

Several promising approaches to deliver HIV antigens (both peptides and recombinant glycoproteins) through the IN route have been evaluated in the last decade. All these studies used either a potent mucosal adjuvant, a delivery system or a fusion protein to engender a strong mucosal and humoral response to HIV antigens.

Staats *et al.* [5,32] demonstrated that IN administration of a HIV-1 peptide vaccine (T1SP10 MN) with cholera toxin (CT) in mice, induced significant levels of antipeptide serum IgG titres and HIV-1MN neutralising responses. Vaginal antipeptide IgG and IgA titres were also induced. In this study, vaginal HIV-1 IgA was associated with a secretory component, suggesting transepithelial transport of IgA into vaginal secretion. In another study, they also demonstrated that the IN route was superior to the vaginal, gastric or rectal route of immunisation for the induction of systemic and mucosal anti-HIV-1 peptide responses. The use of CT as a mucosal adjuvant is well established with several different antigens, and it has been explored both orally and IN [33].

Another potent mucosal adjuvant that has been evaluated with HIV antigens is the genetically detoxified mutants of heat-labile enterotoxin (LT) from *Escherichia coli* (LTK63, LTR72). Neidleman *et al.* [34] demonstrated that IN immunisation of HIV-1 gagp55 antigen with these mutants (LTK63, LTR72) induced CTL responses that were comparable to those obtained by IM immunisation of the same formulation. Furthermore, LTR72 induced both local (mesenteric LNs) and systemic (spleen) CTL responses after oral immunisation of the formulation. Morris *et al.* [35] also demonstrated that another LT mutant, LT (Arg192Gly), induced both serum antibodies and CTL responses against HIV-gp160 and E7 peptide.

Recently, Manocha *et al.* [36] have shown that the use of a plant lectin (UEA-1) along with a gp41 peptide delivered IN within poly(lactic-co-glycolic acid) microparticles induces both enhanced serum antibodies and CTL responses to the peptide. The use of C poly G nucleotides (CpG) as an adjuvant for IN immunisation with gp120, either in combination or as a conjugate has been reported by Horner *et al.* [37]. The IN immunisation resulted in a strong serum antibody

response to gp120 and also a strong secretory IgA response in the vaginal washes and fecal samples.

Two groups [38,39] have evaluated the use of various cytokines (IL-1a, IL-12 and IL-18) as mucosal adjuvants with a HIV-1 gp120 and a HIV-1 env peptide immunogen (C4-V3). They reported responses after IN immunisation; the peptide and cytokines were similar to those seen with the peptide and CT as an adjuvant. The formulation with the cytokines was effective at inducing potent peptide-specific mucosal and systemic responses.

Borsutzky *et al.* [40] have reported the use of a synthetic lipopeptide (macrophage-activating lipopeptide-2 [MALP-2]) in combination with a HIV-1 Tat protein as a suitable formulation that can be administered IN and induces strong serum anti-Tat responses. Similarly, Sakaue *et al.* [41] reported that a liposomal formulation containing encapsulated gp160 in haemagglutination virus can induce strong CTLs and neutralising antibody responses after IN immunisation in mice.

Fusion proteins of gp41 fused to a fragment of influenza virus HA2 haemagglutinin protein was also effective in inducing strong serum and secretory responses against gp41 after IN immunisation [42]. Matoba *et al.* [43] recently reported that a chimeric protein of gp41 peptide and B subunit of CT results in strong serum and mucosal responses after IN immunisation against the gp41 peptide.

IN delivery of recombinant HIV antigens along with a mucosal adjuvant carries a lot of potential for generating potent local and systemic responses, either in combination with other systemic routes (e.g., IM or subcutaneous) or by itself.

4. Intranasal delivery of DNA vaccines against HIV

The use of a plasmid DNA encoding various HIV antigens (env gp120, gp140 and gag p55, gagpol and so on) has also been extensively evaluated along with recombinant proteins as a way of boosting strong systemic and mucosal responses against HIV. The earlier mucosal DNA studies were less encouraging as neither the effective dose nor a suitable delivery system was identified. But in the late 1990s, several papers were reported that demonstrated effective delivery of DNA to induce strong anti-HIV-1 responses.

The first report of the induction of both systemic and mucosal responses with a DNA vaccine administered IN (using cationic lipids to form liposomal complexes) was published by Klavinskis *et al.* [44]. These complexes showed that this liposomal formulation was capable of inducing a strong systemic and mucosal response to a model luciferase protein being encoded by the DNA. Sasaki *et al.* later showed that IN immunisation of naked DNA with monophosphoryl lipid A induced strong systemic responses against HIV-gp160 antigen [45]. The same group also reported that a viscosity enhancer and a bioadhesive polymer, carboxymethylcelluose, when combined with a DNA encoding for HIV-1 gp160, was capable of inducing a strong antibody and CTL response in mice after IN immunisation [46].

Other groups, meanwhile, also explored the possibility of combining more than one DNA encoding for the mucosal adjuvant along with the gene of interest. Xin *et al.* [47] reported that plasmids encoding macrophage inflammatory protein-1α, when given along with a plasmid encoding for HIV-1 pCMV gp 160IIIB markedly enhanced mucosal secretory IgA titres.

Singh and colleagues were the first to report the development and use of a novel cationic poly(lactic-co-glycolic acid) microparticle formulation prepared using a cationic surfactant cetyltrimethylammonium bromide to adsorb DNA for efficient delivery. After the first report with this formulation through the systemic route [48], they then evaluated this formulation with a HIV-1 pCMV gag DNA administered IN [49]. Gag-specific T cell and antibody-mediated responses in local, as well as systemic lymphoid tissues were found. The IN immunisation with this formulation also induced prolonged expression in local and systemic lymphoid tissues in mice.

The use of poly-L-lysine/DNA complexes as a mucosal formulation was reported by Wang *et al.* [50]. They tested three forms of HIV-1 env antigens (gp160, cytoplasmic gp140 and secreted gp140) with poly-L-lysine given IN. The formulation was more effective than soluble DNA in inducing env-specific CTL responses in the lungs, lower respiratory LNs, cervical LNs, submaxillary gland LNs and spleens. At least three doses were required for an optimal response. The use of chitin microparticles along with HIV DNA given IN was also reported [51]. This formulation induced strong viral-specific immunity in mice. Liposomal formulations, such as vaxfectin, have also been shown to be a suitable IN formulation for generating strong HIV-2 env-specific responses in mice [52]. This liposomal formulation was more successful than chitosan microparticles in this report. Overall, IN immunisation of plasmid DNA encoding the antigen of interest is being explored more as a combination with other routes (e.g., IM or subcutaneous).

5. RNA-based vaccines

Anyone who has purified RNA can attest to its labile nature, as there are abundant RNases ready to denature RNA everywhere. Thus, the idea of RNA-based vaccines may intuitively seem problematic. However, several RNA delivery systems have been invented that result in the infection of target cells and delivery of RNA encoding the gene of interest. Therefore, in general, such an approach has a clear advantage over the more popular plasmid DNA immunisation, as RNA, unlike DNA, does not require access to the nucleus and thus minimises the possibility of chromosomal integration [53].

A number of RNA delivery systems as potential vaccine candidates have been described, including purified

cDNA-transcribed RNA, neuraminidase-deficient influenza A virus, tick-borne encephalitis virus, *Listeria monocytogenes*, non-transmissible Sendai virus, liposome-entrapped mRNA, and alphaviruses [53-61]. Of these, the most popular RNA delivery systems for an anti-HIV vaccine have been alphaviruses, either as replicating or nonreplicating replicon particles.

Alphaviruses, including Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus are enveloped RNA viruses that have been developed into replication-defective 'suicide' vectors [62,63]. Alphavirus replicon RNA vectors maintain the nonstructural protein gene and the *cis* replication sequences that are required to drive abundant expression of heterologous antigens from the viral subgenomic 26S promoter; however, these are devoid of any alphaviral structural protein genes required for propagation and spread. These vectors also offer the prospect of natural adjuvanticity and stimulation of the innate immune response, in addition to the antigen-specific adaptive response arising from the cytoplasmic amplification of the vectors through double-stranded RNA intermediates [64]. Replicon vectors have been widely evaluated as vaccine immunogens, both as plasmid DNA replicon vaccines and as virus-like replicon particles [65].

Replicating Venezuelan equine encephalitis virus expressing HIV-1 matrix/capsid were used to inject mice subcutaneously, which induced IgA as well as CTL responses [66]. Cynomolgus macaques immunised parenterally with SFV RNA vectors expressing HIV-IIIB gp160 and challenged parenterally with SHIV-4 were not protected against high viral loads [67], even though a mouse study suggested that compared with a plasmid DNA encoding HIV-env (or HIV-env protein), SFV expressing HIV-env induced the highest serum anti-env antibodies [68]. Sindbis virus based replicon particles, either alone or in the form of chimeric replicon particles with Sindbis outer structure and Venezuelan equine encephalitis virus RNA replicon particles, have been successfully used to induce protective responses in mice [20,29] and in rhesus macaques [69] Thus, alphaviruses given IN hold promise as a viable vaccine delivery platform against HIV.

6. Live attenuated virus-based vaccines

In general, live attenuated (LA) viruses as antigen-delivery systems offer relatively high potency. However, most have the problem of inducing high antivirus vector immunity, thus making their multiple or even subsequent use obsolete. In this regard, a report using a mouse model indicated that mucosal vaccination (which in this case was IR) overcomes the barrier (i.e., immunity against the vaccinia vector) to recombinant VV immunisation caused by pre-existing poxvirus immunity [70]. If the LA virus is the infectious virus with attenuations to eliminate or reduce disease, there have been clear examples of these reverting to the wild type phenotype/genotype and causing severe disease [71-74]. The level of attenuation seems to inversely correlate with potency as the more attenuated strains are less immunogenic [75,76]. Some examples of LA viruses given IN or through other routes to induce anti-HIV immune responses are provided below.

VV-based LA vaccines have been extensively used to induce anti-HIV responses through IN immunisations alone, or as priming or boosting immunisations. IN priming with HIV-env-expressing influenza virus, and IN boosting with HIV-env-expressing VV in mice, induced systemic cellular responses in the spleen and local responses in the genitorectal-draining LNs [77]. IN priming with DNA and IN boosting with VV-expressing HIV-env induced mucosal and systemic humoral and cellular responses [78]. In a non-human primate model, IN, IM and IR immunisations with a LA pox virus (NYVAC) expressing an immunodominant CD8+ CTL gag-epitope, induced CTL responses in the small intestine [79]. An IL-2-augmented DNA IN-prime/VV IN-boost induced mucosal and systemic humoral and cellular responses, and protected the patient from disease (i.e., all became infected but maintained CD4+ cell counts and did not develop AIDS) [80].

A popular approach for using vaccinia-based anti-HIV vaccines has been DNA priming followed by vaccinia boosting, in effect avoiding strong antivaccinia immune responses. However, except for the papers listed above, the other reports have used systemic or non-nasal mucosal routes for priming or boosting immunisations. A DNA prime/vaccinia boost immunisation containing multiple HIV genes controlled viral loads following IR challenge [81]. A tat/rev/nef-based DNA prime/tat/rev/nef vaccinia boost regimen induced rectal CTL, and controlled acute phase, but not long-term, SIVmac239 viral loads [82]. This lack of long-term protection may have been due to the induction of acute but not memory T-cell responses [83]. A SHIV DNA/VV rectal vaccination of rhesus macaques induced inconsistent cellular but mucosal and systemic humoral responses, and a delayed progression to AIDS [84]. To further enhance the immunogenicity of the DNA prime/vaccinia boost model, plasmid chemokines and colony-stimulating factors have been shown to be effective [85].

Several problems have been reported with regards to the use of vaccinia-based anti-HIV vaccines. Gender differences have been found in mice for the induction of HIV-specific CD8+ responses in the reproductive tract and colon, following IN-peptide priming and VV boosting immunisations. Such a regimen induced strong responses in female but not in male mice, and both priming and boosting with the VV-based vaccine was required to induce optimal responses in the male mice [86]. It has also been reported that small pox vaccine does not protect macaques with AIDS from a lethal monkey pox virus challenge, making the use of a therapeutic vaccinia-based vaccine improbable [87]. It has also been shown that the *nef* gene of HIV and SIV, which is important for pathogenicity and maintenance of high virus loads, is a negative attenuating and cell-to-cell infection factor for VV, hence prohibiting the use of a *nef* gene-expressing VV as an anti-HIV vaccine [88]. It was

recently shown in humans that a *nef* gene-expressing VV was used for three vaccinations on AIDS patients on highly active antiviral therapy (HAART), after which the therapy was interrupted. However, although strong anti-VV and some anti-nef responses were detected, the viral loads rebounded in all patients but, as the CD4+ counts have remained above the pre-HAART levels, 6 out of 14 patients have remained off HAART, offering some hope for this approach [89]. Recent reviews discuss the use of VV in general for HIV vaccines through all routes of immunisations [90-92].

Another LA virus used for IN immunisation against HIV is vesicular stomatitis virus (rVSV). Combination of parenteral and mucosal immunisations with LA rVSV was shown to prevent AIDS in rhesus macaques [93]. A comparison of IN and IM routes of immunisation with rVSV in rhesus macaques demonstrated that the IN route induced higher cellular as well as nasal and saliva, responses; but both routes of immunisation conferred protection against vaginal SHIV challenge [94]. The cellular responses were not measured in the vaginal mucosa and the humoral responses at this site were low after IN immunisations, making deciphering the correlates of protection unallowable. However, it was shown that IN immunisation was as good as, or perhaps better than, IM immunisations for protection against AIDS. However, in another study intra-peritoneal (another systemic route of immunisation) versus IN immunisations with rVSV, expressing HIV-gag and -env, induced far higher CD8+ CTL responses in spleens [95]. Although these studies indicate that rVSV may serve as a vaccine delivery system for a potential anti-HIV vaccine, serious potential hazards exist with regards to this virus. VSV belongs to the family of *Rhabdoviridae*, and causes severe vesiculation and/or ulceration of the tongue, oral tissues, feet and teats of horses, pigs and cattle (symptoms that are indistinguishable from foot and mouth disease), and as an important zoonotic pathogen, can cause disease in humans [96,97]. Therefore, the use of this vector as an ultimate human vaccine delivery system against HIV seems unlikely.

Adenoviruses (Ad), proposed to be used as a vaccine delivery system for anti-HIV vaccines are icosahedral, nonenveloped, double-stranded DNA viruses belonging to the family *Adenoviridae* and infect all mammalian species [98]. Most of the human and non-human primates have pre-existing antibodies against the majority of Ad. Ad are responsible for ~ 5% of upper respiratory tract infections and 8% of childhood pneumonia incidences [99], have caused major outbreaks in the past [99] and emergent strains are on the rise in North American populations [100,101]. There are six subgroups (A – F) and several serotypes within each subgroup (e.g., Ad1, Ad2, Ad5, A6 are in subgroup C). Each serotype is known to cause a different disease or a specific severity of a disease [102]. Other safety concerns stem from the fact that most Ad bind specifically to liver parenchaymal cells [103], and localise to the CNS following IN immunisations [104]. Indeed, administration of the popular Ad5 resulted in the death of a patient [105]. Nonetheless, optimism that the strains used as vaccine delivery vehicles are not disease-causing or only cause minor symptoms, as well as the use of replication defective vectors, have kept Ad-based vaccines as viable vaccine candidates.

Ad-based vaccines have been considered as a vaccine delivery system for anti-HIV vaccines for over a decade [106-107]. In early reports, Ad-expressing HIV-env induced serum HIV-neutralising antibodies following intra-tracheal immunisations of dogs [106]. This study was soon followed by two chimpanzee studies using IN immunisations as stand alone, or as boosting, modality with Ad-expressing HIV-gag and -env inducing variable mucosal and systemic humoral responses [107,108]. To avoid pre-existing immunity, it has been suggested that DNA priming followed by Ad boosting would enhance the responses [111,112]. In addition, priming with Ad and boosting with HIV-env have been reported to reduce acute viraemia following challenge [113,114]. Several reports have also demonstrated immunogenicity of replication incompetent Ad vectors as anti-HIV vaccine vectors [110,114,115]. Thus, in general, although replication-competent Ad vectors pose some safety concerns, they can induce mucosal and systemic humoral responses following IN immunisations as a stand alone modality, as well as induce strong CTL responses following parenteral immunisations as a stand alone or as boosting modality. The use of replication-incompetent Ad vectors could circumvent the safety issues but may induce significantly reduced immune responses, which may nonetheless be sufficient to reduce viraemia.

Influenza virus binds to the epithelial cells of the upper and lower respiratory tract and induces a relatively strong immune response against itself. Therefore, using an influenza virus as a mucosal vaccine delivery vector may be feasible, although again safety concerns do prevail and thus far the mucosal use of such vectors has not been reported in primate models. Recombinant influenza A virus containing a HIV-nef insert that did not replicate in the respiratory tract, was used for a single IN immunisation of mice and induced CD8+ T-cell responses in the spleen, in the LNs draining the respiratory tract and in the urogenital tract [116]. Moreover, a chimeric influenza virus that did replicate in the respiratory tract of mice, containing a HIV-env neutralising epitope, induced humoral responses in the spleen, lungs and urogenital tracts [117,118]. Interestingly, IN immunisation with inactivated influenza virus enhanced immune responses to coadministered SHIV virus-like particles (VLPs), suggesting that influenza virus viability may not be required for the adjuvant action of influenza viruses [119]. Other LA virus approaches for mucosal immunisation against HIV include the use of a rhabdovirus (rabies virus), polio virus and a plant virus (cowpea mosaic virus), which have induced mucosal and systemic humoral and cellular responses [120-122].

7. Inactivated bacteria-, inactivated virus- and virus-like particle-based vaccines

The idea of attenuating the HIV-1 virus is impractical due to the obvious issues with reverting to the wild type and causing

disease. In addition, inactivating the HIV-1 virus would pose the risk of a few viruses escaping inactivation and also causing disease. For both LA and inactivated HIV-1, large-scale production will require a very high biological safety level facility, which again would be impractical. Nonetheless, inactivated HIV or SHIV-capturing nanoparticles have been produced and used to induce vaginal antibody responses in mice and rhesus macaques following IN immunisations [123,124]. Moreover, inactivated HIV-1 plus CpG adjuvant induced genital CTL and antibody responses in mice, which were subsequently protected against vaginal challenge with recombinant VV [125,126]. There have also been examples of IN immunisations with recombinant bacille Calmette–Guérin bacteria encoding a HIV-1 antigen [127], or a heat-inactivated bacteria conjugated to HIV-env antigen [128]. 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 the serious safety concerns.

VLPs are another candidate for an anti-HIV vaccine. VLPs are produced by transfecting eukaryotic cells, yeast, insect or mammalian cells, with DNA encoding the gene of interest, usually delivered by baculovirus or VV. The VLPs are then formed, without a genome, in the cell and are either secreted or remain in the cell to be purified. VLPs made of HIV-gag alone or with HIV-env expressed on the surface of gag-VLPs have been developed and used for IN immunisations in animal models [129,130]. As a different approach, IN immunisations of mice with chimeric influenza HA/SHIV VLPs induced mucosal and systemic humoral and cellular responses [131]. An inherent problem with most studies using baculovirus-based VLPs is that no, or insufficient, efforts were made to purify VLPs in the absence of baculovirus. Baculoviruses induce innate antiviral effects and can clearly enter mammalian cells and, although they do not replicate, the DNA enters the nucleus, thus raising concerns about host cell chromosomal integration [132].

8. Live bacterial delivery systems

Immunisation with invasive bacterial systems such as *Shigella*, *Salmonella* and *Listeria* as a vector system for effective gene transfer into the cytoplasm of infected cells has been explored by several groups in the last few years [133]. The use of a LA bacterial delivery system as an effective modality for generating strong responses to HIV antigens after IN immunisation has also recently been explored along with the other approaches described above.

The first documented report of HIV protective immunity with a live *Shigella* DNA vector was published by Shata *et al.* [134]. A single IN dose of *Shigella*/HIV-1 gp120 vaccine vector in mice induced a strong CD8 T-cell response comparable to systemic immunisation with a vaccinia-env vector [134]. In addition, this single immunisation was also effective in affording protection against a vaccinia-env challenge. The same group showed that this vector was also effective orally in mice [135]. Vecino *et al.* published their findings with attenuated *Shigella* and *Salmonella* delivering HIV-1 gp120 IN in mice [136]. They reported that attenuated *Shigella* was much more effective than attenuated *Salmonella* in generating CD8+ T-cell responses after a single IN immunisation. The IN route was more effective than the IM route for generating higher IgA titres in the vaginal washes. Xu *et al.* [133] reported that a single IN dose of *Shigella flexneri* 2a mutant encoding for HIV-1 SF2 gag was effective in inducing gag-specific T-cell responses both in the spleen and the lungs. The live bacterial delivery as an approach is currently being explored more through the oral route than the IN route, for ease of administration. This delivery approach has not received much attension for IN immunisation in the last decade or so.

9. Potential problems associated with intranasal immunisation

The advantages of IN immunisations , delivery systems and adjuvants have been discussed (Figure 3); however, the potential risks also need to be considered. In late 1990s, Berna Biotech of Switzerland used an inactivated influenza vaccine containing haemagglutinin and neuraminidase antigens in liposomes in combination with wild type LT from *E. coli*. Shortly after IN immunisations with this formulation, a strong association between the vaccination and increased cases of Bell's palsy was observed [137] and the vaccine was removed from the market. It was then deduced that the enterotoxin used as an adjuvant may be responsible for the Bell's palsy [138], a form of facial paralysis that in most cases is short lived [139]. Later, it was found in a mouse model that CT, an enterotoxin resembling LT, redirected coadministered protein antigens into the olfactory bulbs and nerves, following IN administration [140]. CT, as with LT, binds to GM1 monogangliosides, which are abundant in the CNS. In fact, the neuronal connections between olfactory nerves and epithelium and the olfactory bulb are used by some pathogens to reach the CNS and the brain [140]. Later, however, it was shown that in mice a nontoxic mutant of CT, CTA1-DD, given IN did not cause inflammation or accumulate in the nervous tissues [141]. This work was recently followed up to show that enterotoxin-based adjuvants require both the ADP-ribosyltransferase activity, as well as GM1-binding ability in order to cause antigen redirection into the olfactory neuroepithleium [142]. Finally, it was shown in human trials that a mutant of LT, LTK63 with undetectable ADP-ribosyltransferase activity, was safe and acted as an adjuvant with a trivalent subunit influenza vaccine for IN immunisations [1]. Thus, although the IN route of immunisation holds great promise for the induction of immune responses in the respiratory and genital tracts as well as systemically, caution is needed in selecting the vaccine contents.

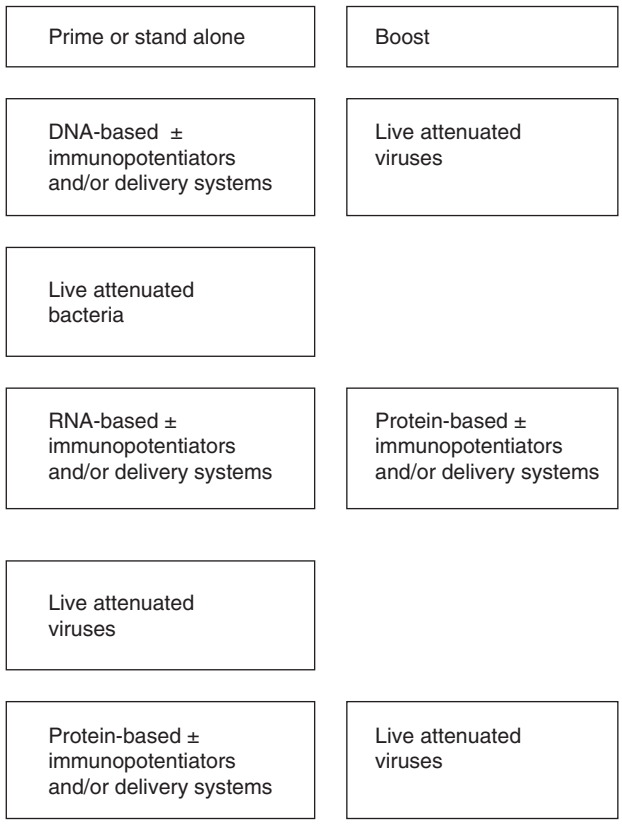

Figure 3. Current approaches to intranasal immunisations against HIV as priming, boosting, or stand alone mode of immunisation.

10. Expert opinion

The first issue to consider regarding IN immunisations against HIV, or any other pathogen, is whether to administer the vaccine IN (e.g., using a nasal spray) or intra-tracheally, through other devices. There is enough evidence to suggest that IN immunisations would induce vaginal responses to warrant testing this immunisation route in humans. However, important safety issues should be considered, such as the possibility of the vaccine reaching the brain through the olfactory bulb. As such, the choice of the vaccine delivery vehicle becomes ever more important. In addition, as perhaps the most important aspect of vaccination is the induction of long-term immunological memory, more studies are needed to determine the importance of IN versus intravaginal or other routes of immunisations for long-term protection against HIV. Therefore, long-term memory studies, particularly in non-human primates, are lacking. It is also imperative to determine what type of innate and adaptive immune responses are required to induce long-term protection against vaginal versus systemic transmission of HIV. Regardless of the route of transmission, HIV or SIV cause rapid depletion of CD4+ T helper cells in the mucosal effector sites of the gastrointestinal, respiratory and genitourinary tracts. Thus, because it is unlikely that an IN vaccine can inhibit the HIV virus to reach all of these sites, an effective vaccine should be devised to eliminate the virus at virtually all mucosal effector sites. However, although IN immunisation will most likely be effective in the respiratory and the genitourinary tracts, it may not induce effective responses in the gastrointestinal tract. This is an important issue that needs to be investigated. If this turns out to be the case, IN immunisation needs to be combined with oral immunisations to induce immune responses at the relevant mucosal tissues. But, as previously discussed, induction of immune responses in the gastrointestinal tract through oral immunisations is generally more difficult compared with other routes of immunisation. This is because significantly more antigen, or special precautions to protect the vaccine against enzymatic or acidic degradation in the stomach or the intestine, will be required. It is possible that IN immunisations have to be combined with parenteral routes of immunisation to enhance both mucosal and systemic immune responses and, although such approaches have been reported in a few studies, further investigation in this area is warranted. Finally, as various vaccine delivery systems or immunopotentiating adjuvants induce various innate and adaptive responses, it is plausible that an IN-based vaccine against HIV will require a combination of two or more vaccine modalities in order to induce both CTL and broadly reactive neutralising antibodies.

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