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Review

New insights in mucosal vaccine development

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ABSTRACT

Mucosal surfaces are the major entrance for infectious pathogens and therefore mucosal immune responses serve as a first line of defence. Most current immunization procedures are obtained by parenteral injection and only few vaccines are administered by mucosal route, because of its low efficiency. However, targeting of mucosal compartments to induce protective immunity at both mucosal sites and systemic level represents a great challenge. Major efforts are made to develop new mucosal candidate vaccines by selecting appropriate antigens with high immunogenicity, designing new mucosal routes of administration and selecting immune-stimulatory adjuvant molecules. The aim of mucosal vaccines is to induce broad potent protective immunity by specific neutralizing antibodies at mucosal surfaces and by induction of cellular immunity. Moreover, an efficient mucosal vaccine would make immunization procedures easier and be better suited for mass administration. This review focuses on contemporary developments of mucosal vaccination approaches using different routes of administration.
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1. Introduction

The epithelial lining of mucus membranes covers an area of several hundred square metres in an adult. Mucosal surfaces are mainly represented by the gastrointestinal, the respiratory and the urogenital tracts and therefore are vulnerable to infection by pathogenic microorganisms. Mucosal surfaces are protected from external attacks by physicochemical defence mechanisms, innate and adaptive mucosal immune systems which are designed to distinguish antigens that enter the body through mucosal surfaces from those introduced directly into the bloodstream. The mucosal immune system can principally be divided into inductive and effector sites. Antigens are sampled from mucosal surfaces either through collaboration with professional antigen-presenting dendritic cells (APCs), or by producing a specialized epithelial phenotype, the M cell and then stimulate cognate naive T and B lymphocytes [1]. Epithelial barriers on mucosal surfaces at different sites in the body differ dramatically in their cellular organization, and antigen sampling strategies at diverse mucosal sites are adapted accordingly. Multilayered squamous epithelia line the oral cavity, pharynx, esophagus and urethra whereas the intestinal mucosa is covered by only a single cell layer, and the airway and vaginal lining varies from pseudo-stratified to simple epithelium. These diverse epithelia are not impenetrable barriers, but rather are cell assemblies that control cross-talk between the lumen and the lamina propria using multiple antigen sampling strategies. In stratified and pseudo-stratified epithelia, antigen-processing

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dendritic cells serve as motile "scouts" that move into the epithelium, obtain samples of luminal antigens, and migrate back to local or distant organized lymphoid tissues. In simple intestinal and airway epithelia whose intercellular spaces are sealed by tight junctions, specialized epithelial M cells deliver samples of foreign material by transepithelial transport from the lumen to organized lymphoid tissues within the mucosa [2,3].

At the immune effector sites level, such as *Lamina propria* (LP), where the effector cells locally controlling foreign agents, secretory antibodies are induced, especially secretory IgA (SIgA) but also IgM. IgA is the major isotype in secretions, the most important being those of the epithelium lining the intestinal and respiratory tracts whereas IgG is the principal isotype in the blood and extracellular fluid [4]. IgG effectively opsonises pathogens for engulfment by phagocytes and activates the complement system. In contrast IgA is a less potent opsonin and a weak activator of complement. IgA operates mainly on epithelial surfaces where complement and phagocytes are absent, and therefore IgA function chiefly as a neutralizing antibody. Animal viruses or bacteria infect cells or host by binding to a particular cell-surface receptor, often a cell-type-specific protein that determines which cells they can infect (virus) or cell-surface molecules called adhesions that enable them to bind the surface of host cells (bacteria). Antibodies against those patterns can inhibit these adhesive reactions and prevent infection. IgA antibodies secreted onto the mucosal surfaces of the intestinal, respiratory, and reproductive tracts are particularly important in preventing infection by inhibiting the adhesion of bacteria, viruses, or other pathogens to the epithelial cells lining these surfaces [5]. The adhesion of bacteria to cells within tissues can also contribute to pathogenesis, and IgG can protect from damage as IgA protect at mucosal surfaces.

IgA-secreting plasma cells are found predominantly in the LP. At this level, IgA can be transported across the epithelium to its external surface and are secreted as a dimeric IgA molecule associated with a single J chain. This polymeric form of IgA can be endocytosed at the basolateral surface by the poly-Ig receptor (pIgR), then transcytosed and finally secreted into the lumen, where it can combine with antigen to form immune complexes. The extracellular portion of the pIgR still attached to the Fc region of the dimeric IgA may help to protect it from degradation [6]. The neonatal Fc Receptor (FcRn) plays also a role in mucosal immunity during the passive delivery of IgG from mother to young via the placenta or the intestinal route. In adult, it can also transport IgG across mucosal surfaces to confer resistance to intestinal pathogens and therefore use to deliver antigens fused with an IgG Fc fragment through mucosal surfaces [7].

Mucosal associated lymphoid tissue (MALT) is the principal mucosal inductive site where immune responses are initiated. LP is considered to be an effector site which is also important for expansion and terminal differentiation of B cells. MALT comprises approximately 80% of immune cells in the body and is the largest lymphoid system in mammals [8]. It has three major functions: (1) the protection of mucosal surfaces against colonization and invasion by microbial pathogens, (2) the prevention of the internalization of commensal bacteria or antigens as non-degraded proteins derived from food and environment, and (3) induction of tolerance against innocuous soluble substances, as well as commensal bacteria. Mucosal effector sites are formed by a surface epithelium with a concentration of intraepithelial T lymphocytes (IEL) and secretory antibodies (especially SIgA). Sub-epithelial compartment, or chorion, is an effector site where pile up effector cells (NK-like cells, macrophages, B and T cells). Antigen presenting cells (APC) including dendritic cells (DCs), sentinels of the immune system, are also present in the mucosal lymphoid tissue, to detect foreign agents.

It should be notified that mucosa are naturally highly exposed to huge amount of antigens every days so different regulation mechanisms exist in a manner that does not result in untoward immune reactions. Those mechanisms are called immune tolerance and depend of the dose of antigen: anergy/deletion (high dose) or regulatory T-cell (Treg) induction (low dose). It has been shown in mice that tolerance to oral antigen requires CD8+ T cells for local suppression of IgA responses [9]. In contrast, recognition of foreign agents as pathogens requires the recognition of pathogens-associated molecular patterns (PAMPs), like LPS or flagellin. PAMPs are danger signals which are recognized by pathogen recognition receptors (PRRs) like Toll-like receptors (TLR) or Nod-like receptors (NLR) expressed by cells of the innate immune system and present in quantities at mucosal sites both in animal models and in humans. The role and the high expression of NOD1 receptor have been described in lungs during asthma [10] and in intestinal mucosa for inflammatory bowel diseases (IBD) [11]. NOD2 receptor is also involved in IBD and expressed on the intestinal mucosa of Crohn patients [12]. TLR7 expression has been described in the human intestinal mucosa as TLR4 has been shown to be expressed at the genital level.

It's now well described that local mucosal immune responses are important for protection against diseases which occur mainly by those routes. Topical application of a vaccine may be necessary to induce a protective immunity. In some cases, systemic IgG are sufficient to protect against occurring mucosal infections such as poliovirus. Mucosal vaccines could induce in certain conditions with the use of appropriate adjuvants, both systemic IgG, protective SIgA and CTL responses against pathogens [13]. By the migration of IgA antibody-secreting cells (ASCs), local mucosal immunization could lead to antigen-specific IgA production at distant mucosal sites [14]. In contrast, traditional injected vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infections at mucosal sites [4,15]. In a practical way, they are easily administered (e.g. oral route), and therefore more accessible to developing countries. As soluble antigens are not efficiently uptake when administered by mucosal routes, and generally induce immune tolerance, mucosal immunization requires adjuvants and/or efficient carrying vehicles as delivery systems. The ideal mucosal vaccine should: (1) preserve vaccine antigens from enzymatic or chemical degradation (2) limit their elimination or excessive dilution in organism, (3) facilitate the preferential uptake of antigen by specialized NALT/GALT/BALT M cells in order to target APC, dendritic cells or epithelial cells, (4) facilitate the co-uptake of both antigen and adjuvant to APCs in order to stimulate appropriate specific immunity as neutralizing SIgA and/or helper and cytotoxic T lymphocytes. Secretory antibodies may block the colonization of the mucosal epithelium by pathogens or prevent attachment of microbial toxins on epithelial cells, and then cytotoxic T cells could eliminate infected cells and prevent microbial invasion.

This review focuses on new vaccinal approaches using different mucosal routes to induce appropriate mucosal and systemic immune responses. At first, we describe the different mucosal vaccines that are currently used in clinical practice and then we will develop different approaches to improve the effectiveness of mucosal vaccination.

1.1. Registered human mucosal vaccines (Table 1)

Only seven vaccines are routinely administered mucosally to humans. They target five of the main enteric pathogens (Table 1): poliomyelitis, *Vibrio cholerae*, *Salmonella typhi*, rotavirus, and influenza whereas vaccines are still lacking against the two other most important causes of enteric diseases, enterotoxigenic *Escherichia coli* (ETEC) and Shigella.

Poliomyelitis is due to poliovirus which enters the organism through oral route and cross the intestinal epithelial lining through M cells and enterocytes. In approximately 0.1–2% of cases, the virus

Table 1
Licensed vaccines against mucosal infections.

Infection	Vaccines	Route of immunization	Commercial name/producer	Reference
Poliomyelitis	Inactivated polio vaccine	Subcutaneous/intramuscular	Salk vaccine	[16]
	Live attenuated polio vaccine	Oral	Many	[18–20]
Cholera	Cholera toxin B subunit & inactivated *V. cholerae* 01 whole cells	Oral	Dukoral (SBL Vaccin)	[21,24]
	Live attenuated *V. cholerae* 01 strain (CVD 103.HgR)	Oral	Orochol (Bern, SSVI)	[25–27]
Typhoid	Vi polysacharide	Subcutaneous/intramuscular	typhimVi (Aventis)	[28,29]
	Ty21a live attenuated vaccine	Oral	Vivotif (Bern, SSVI)	[30]
Rotavirus	Live attenuated monovalent human rotavirus strain	Oral	RotaRix (GSK)	[24,35,36]
	Pentavalent live vaccine	Oral	RotaTeq (Merck)	[37,38]
Influenza	Live attenuated cold-adapted influenza virus reassortant strains	Nasal	Flumist (MedImmune)	[40,41]

invades the nervous system and causes total paralysis in a matter of hours. Two vaccines have been developed against poliomyelitis: the injectable inactivated polio vaccine (IPV) and the oral polio vaccine (OPV). The first one was developed by Jonas Salk in 1952, containing the three serotypes inactivated by formalin [16]. Since 1978, the eIPV form (enhanced) is used for vaccination. This vaccine is administered subcutaneously or intramuscularly three times, and provides systemic immunity that protects vaccinated people. However, the intestinal mucosal response remains low, and this vaccine therefore is not sufficient to prevent the spread of the virus through fecal-oral route. The oral polio vaccine (OPV) is the main type of the classical oral-mucosal vaccines and there is no vaccine that is more amenable to mass immunization. Indeed, it requires only the installation of few drops into the subject's mouth, at whatever age. OPV is a live-attenuated vaccine developed by Sabin [17] and produced by passage of the virus through non-human cells at a sub-physiological temperature, which produces spontaneous mutations into the viral genome. Three doses of live-attenuated OPV induce systemic and mucosal-neutralizing antibodies against the three poliovirus types in more than 95% of recipients. OPV produces a local SIgA immune response in the intestine, the primary site of wild poliovirus entry, which helps to prevent infection against wild type virus in endemic areas [18]. OPV induces also antibodies production in the blood that will protect against myelitis by preventing the spread of poliovirus to the nervous system [19]. This vaccine has also served as a useful tool for elucidating fundamental aspects of mucosal immunity in humans [20]. Today, OPV is recommended for routine immunization in the United States. IPV produces less gastrointestinal immunity than does OPV, and primarily acts by preventing the virus from entering the nervous system. In regions without wild-type poliovirus, inactivated polio vaccine is the vaccine of choice because of the reduced risk of exposure and the continued occurrence of vaccine-associated paralytic poliomyelitis (VAPP) after oral polio vaccine (approximately 1 of 2.5 million OPV doses) [18]. In regions with higher incidence of polio, and thus a different relative risk between efficacy and reversion of the vaccine to a virulent form, live-attenuated vaccine is still used.

V. cholerae causes severe diseases and exerts its powerful diarrheagenic effect via the intoxication of enterocytes with the cholera toxin (CT). Only two serogroups express the CT: O1 and O139. Despite attempts to injectable cholera vaccines, which did not induce significant gut mucosal immune responses, two improved oral cholera vaccines have become available but none in the USA. The most widely used is Dukoral™, which is given by mouth. Each dose contains approximately 2.5×10^{10} inactivated *V. cholerae* O1 of Inaba and Ogawa serotypes (classical & El Tor biotypes) and the recombinant non-toxic B-subunit of cholera toxin (CTB). Dukoral™ has been proved to be safe and stable with a large efficacy and effectiveness [21]. A second non-living cholera vaccine, Shanchol™ was recently licensed in India [22,23] and contains killed-whole-cell *V. cholerae* O139 and O1 and is not combined with CTB.

Protection is essentially mediated by local production of antitoxin and antibacterial SIgA antibodies in the gut [24]. Today, it is available in over 60 countries. The second internationally licensed oral cholera vaccine is the live attenuated vaccine CVD 103-HgR, containing a genetically manipulated classical *V. cholerae* O1 Inaba strain with a deletion in the gene encoding the cholera toxin [25]. The vaccine has been proved to be safe and to generate immune responses (blood IgG and IgA antitoxin) in more than 72% of healthy volunteers from industrialized countries [26]. Unfortunately, its efficacy has not been demonstrated in clinical trials on a large scale in endemic areas (Indonesia), perhaps due to antigenic dose too low and too weak to affect cholera during the study period [27]. Originally registered in Switzerland, it is currently not available. Despite oral cholera whole-cell killed vaccines (Dukoral™) can provide significant protection, further efforts are still required to provide more effective protection of young children.

Typhoid fever is a serious systemic infection, caused by the enteric pathogen *Salmonella enterica* serovar Typhi, a highly virulent and invasive enteric bacterium and characterized by persisting high fever. Two new-generation of typhoid vaccines have replaced the old, reactogenic inactivated whole-cell vaccine used in the past. These new-generation vaccines – live, oral Ty21a and injectable Vi polysaccharide – have been shown in large-scale clinical trials to be safe and efficacious, and are internationally licensed for people aged >2 years [28]. The Vi capsular polysaccharide vaccine (ViCPS) is the most generally used vaccines recommended by the World Health Organisation (WHO). This vaccine is injected either under the skin or into a muscle at least seven days before travelling to the typhoid-affected area. Typhim Vi™ (Sanofi-Aventis) is well tolerated and is assumed to protect by way of serum antibodies [29]. The mucosal licensed vaccine against typhoid fever is the orally administered live attenuated Ty2 strain of *S. typhi*. It was developed in the early 1970s in which multiple genes, including the genes responsible for the production of Vi, have been mutated chemically. Ty21a is well tolerated and two formulations were licensed: enteric-coated capsules and a liquid formulation. In two trials in school-aged children in Chile, the enteric-coated capsule version was found to be 33.2% protective after 36 months, 67% after 3 years and 62% after 7 years, whereas the liquid formulation was 77% protective after 3 years and equally protective after 7 years of observation [30,31]. Those studies demonstrated that the formulation, the number and the spacing between the doses administered influence the level of protection. It has been shown that oral immunization with Ty21a induces mucosal bacterial antigens specific sIgA and systemic IgG against the O polysaccharide. Moreover, Kantele et al., shown that oral, but not parenteral, typhoid vaccination induces circulating antibody-secreting cells that all bear the $\alpha 4\beta 7$ which guides the lymphocytes to home to the gut mucosa [32]. Those responses are responsive for the protective effect of the oral vaccine.

Rotaviruses together with ETEC are the leading cause of severe diarrhea among infants and young children. Each year, more than

Table 2
Oral delivery of vaccines.

Delivery strategies	Examples	Responses	Model	Reference
PLA or PLGA nanoparticles	PLG-encapsulated CS6 antigen from *E. coli*	– IgA antibody-secreting cell responses ↗ – Serum IgG responses ↗	Human	[50]
	Lotus tetragonolobus from Winged or Asparagus pea anchored PLGA nanoparticles	– Mucosal and systemic response ↗	Mouse	[76]
Liposomes	Plasmid DNA pRc/CMV HBS encoding the small region of hepatitis B surface antigen (HBsAg) into Lipodine liposomes	– IgA responses ↗	Mouse	[53]
	L-BLP25 (liposomal formulation of BP25 lipopeptide, MPL® and three lipids)	– Median survival time of 4.4 months longer	Human	[55]
Bacterial ghost	*Escherichia coli* O157:H7 bacteria ghosts	– Cellular and humoral immunity ↗	Mouse	[58]
Plant lectins/adjuvants	Mistletoe lectin 1, tomato lectin, *Phaseolus vulgaris*, wheat germ agglutinin (WGA), and *Ulex europaeus 1*	– Serum and mucosal antibody responses ↗	Mouse	[67,68]
	Rice-based vaccine that expressed cholera toxin B subunit	– Production of specific serum IgG and IgA antibody after three intra nasal or oral doses ↗	Mouse	[65]
Transgenic plants (bioreactors)	Plant-based rotavirus VP6	– High titers of anti-VP6 mucosal IgA and serum IgG ↗	Mouse	[62]
	Recombinant LT-B in transgenic corn	– Both serum IgG anti-LT and numbers of specific antibody secreting cells ↗	Human	[63]

↗: Increase.

500,000 children die from diarrheal disease caused by rotavirus [33]. Most rotavirus strains belong to one of five antigenic groups. A quadrivalent vaccine based on a Rhesus monkey rotavirus strain equipped with human rotavirus genes was licensed for a short time (RotaShield™), but it was discovered that the vaccine may have contributed to an increased risk for intussusceptions, or bowel obstruction, in one of every 12,000 vaccinated infants [34]. Today, two oral vaccines are licensed: Rotarix™ (GlaxoSmithKline) and Rotateq™ (Merck). The first one is a human, live attenuated rotavirus vaccine containing a rotavirus strain of G1P specificity. Rotarix™ is indicated for the prevention of rotavirus gastroenteritis caused by G1 and non-G1 types (G3, G4, and G9) when administered as a 2-doses series in infants and children. This vaccine has shown good safety, and when tested in an efficacy trial in Brazil, Mexico and Venezuela, it conferred 61–92% protection against rotavirus hospitalizations [35]. The licensure of Rotarix™ is in process in the European Union [36]. The other oral attenuated vaccine (RotaTeq™) is also licensed in the United States, Canada, and other countries [37]. This is a pentavalent reassortant bovine-human vaccine generated to contain human rotavirus genes for each of the main rotavirus serotypes (P, G1, G2, G3 and G4). In Finnish infants, three doses of vaccine in different concentrations gave 58–74% protection against any rotavirus disease [38]. Intestinal IgA is probably the most important mechanism for long-term protection against rotaviruses and correlates of protection after vaccination may vary depending on the type of vaccine studied. For some heterologous vaccines, serum antibodies may not provide adequate correlates of protection; for homologous vaccines serum IgA may reflect antiviral immunity. Doubts exist concerning the importance of subtype immunity to rotaviruses and the lack of a clear understanding of the protective immunity is an important limitation of current vaccine development [39].

Influenza vaccines are available either by injection, flu shot of trivalent inactivated vaccine, or nasal spray of live attenuated influenza vaccine, Flumist™. Injectable vaccines work by induction of serum antibodies, mainly IgG, which prevent systemic spread of the pathogen and which may also, through transudation, exert a local protective effect at the mucosal surfaces of the lower respiratory tract. However, this vaccine induces poorly mucosal IgA antibodies and cell-mediated immunity. In contrast, Flumist™ may elicit a long-lasting, broader immune (humoral and cellular) response, which more closely resembles natural immunity [40]. Both mucosal and systemic immunity contribute to resistance to influenza infection and disease. Locally produced SIgA antibodies to virus surface hemagglutinin and neuraminidase are important for protection of the upper respiratory tract and corresponding serum IgG antibodies for protection of the lower respiratory tract and against viremia. Cell-mediated immunity, mainly against virus matrix and nucleoprotein antigens, does not protect against infection, but is important for clearance of viruses and recovery from illness. The nasal vaccine induces significantly higher local IgA antibodies in nasal washings and local cell-mediated immunity but less high serum antibody titers than the injectable vaccine. Despite these differences in immune responses, the two types of vaccine have comparable protective efficacy (60–90%), and in elderly people, their combined use may increase the efficacy compared with the use of either vaccine alone [40]. Flumist was the first and (as of 2007) the only live attenuated vaccine for influenza available outside of Europe using mucosal route. In September 2009, an intranasal vaccine Flumist™ for the novel H1N1 influenza virus was also approved in the United States [41].

To date the number of mucosal registered vaccines for human application remains very limited and are principally based on live attenuated pathogens and/or vectors, which contain their own danger signals and mechanisms of mucosal entry. However, there is a risk of reversion. Subunit vaccines could be a safe alternative but the hurdles are far more daunting than live attenuated vaccines. Many efforts are made to improve the immunogenicity and the safety of mucosal vaccine with a particular emphasis on the use of adjuvants but also and above in the use of new improved routes of administration.

2. Mucosal routes of immunization

2.1. Oral delivery of vaccines (Table 2)

Oral delivery of vaccines is an attractive mode of immunization because of its acceptability and its simplicity of administration. Indeed, as shown before, current internationally licensed mucosal vaccines are predominantly live-attenuated and are mostly administered by oral routes. The GALT play a central role in the induction of oral tolerance and immunity to infectious agents. In PP, DCs are separated from the intestinal lumen by the follicle-associated epithelium (FAE). It allows transfer of pathogens in lymphoid tissue through Microfold (M) cells [42]. Apical surface of M cells has little glycocalyx, presumably aiding antigen uptake, and the

basolateral membrane is heavily invaginated, both shortening the distance of cytoplasm to be traversed by antigens and accommodating the closely associated lymphoid cells. Antigen is then captured by DCs, causing their maturation and their migration to the intrafollicular areas [43]. M cells represent a potential portal for oral delivery of peptides and for mucosal vaccination, since they possess a high transcytotic capacity and are able to transport a broad range of materials. Although the mucosal immune system comprises several anatomically remote and functionally distinct compartments, it is firmly established that the oral ingestion of antigens induces humoral and cellular responses not only at the site of antigen exposure but also in other mucosal compartments [44]. This is due to the dissemination of antigen-sensitized precursor B and T lymphocytes from the inductive to the effector sites.

A lot of oral vaccine development studies are performed in animal models and it is very important to keep in mind that often animal model cannot be extrapolated to human. Indeed, size, number, distribution, and composition of Peyer's patches may vary depending on species or strain. For example, Peyer's patches are generally smaller in Fischer 344 rats than in Wistar rats [45]. Moreover, it is unknown whether human ILFs have properties similar to the murine counterparts. However, irregular lymphoid aggregates, which may have germinal centres (GCs) with follicular dendritic cells, are induced in chronic inflammatory bowel disease lesions [46] although the function of these ILF-like structures remains elusive [47]. Animal vaccines studies should take into account these differences.

Several strategies, shown in Table 2, including the use of biodegradable polymeric particles and liposome, have been tested. Antigens, adjuvants, and targeting molecules could be incorporated individually or in combination into these microparticles. These vehicles may thus act as immune-stimulants while preventing the degradation of immunogens by enzymes in the gastro-intestinal (GI) tract. These particulate formulations might also interact with M cells and release immunogens slowly, consequently promoting phagocytosis. As an example, PLA (poly(lactic acid)) or PLGA (poly(lactic-co-glycolic acid)) nanoparticles are suitable protein carriers offering antigen protection, increased penetration across mucosal surface and controlled release of encapsulated antigen [48]. The efficacy of these microparticles has been tested in several animal studies and in a limited number of clinical trials. Interesting results were revealed in mouse model by their capacity to cross *in vivo* epithelial barriers or to be taken up by mucosal DCs [49]. In humans, oral delivery of PLG-encapsulated CS6 antigen from *E. coli* induces mucosal IgA responses. However, differences between immune responses that were generated by encapsulated and non-encapsulated antigen were not significantly different [50]. Further clinical studies are needed to assess the effectiveness of vector such as PLA or PLGA particles.

Oral delivery of DNA vaccines encoding various antigens has also been evaluated in various animal studies. The pitfall associated with this strategy is the low uptake of DNA from the intestinal tract, which consequently limits B and T cell immune responses [51]. It was also suggested that PLGA nanoparticles were promising carriers for plasmid DNA vaccine and might be used to vaccinate fish by oral approach. Indeed they showed that after oral administration, FITC-labeled PLGA nanoparticles were detected in blood of fish, and RNA containing major capsid protein (MCP) gene information existed in various tissues of fish 10–90 days [52]. Another study has incorporated plasmid DNA pRc/CMV HBS encoding the small region of hepatitis B surface antigen (HBsAg) into Lipodine liposomes. They showed that, after oral immunization of mice, the complex revealed that secreted IgA responses against the encoded HBsAg were substantially higher after dosing with 100 μg liposome-entrapped DNA compared to naked DNA [53]. However, clinical experience has been mixed. Serum antibody titres after oral delivery of liposome-encapsulated tetanus toxoid (TT) or diphtheria toxin (DT) to humans were variable and were lower than those observed for animals [54]. In contrast, the use of L-BLP25 (also known as Stimuvax®, a lyophilized, liposomal formulation of BP25 lipopeptide, MPL® and three lipids) into clinical studies demonstrate a well-tolerated profile and elicits a cellular immune response in patients with lung cancer [55].

Recombinant or attenuated strains of various bacteria such as *Salmonella*, *E. coli*, or *Listeria*, have been also used as vectors to deliver antigens into the GALT [56,57]. These vectors not only target the DNA or peptide vaccine construct to APC, but also provide a strong danger signal, acting as natural adjuvants, thereby promoting efficient maturation and activation of dendritic cells [58]. However, immune responses against the vectors eventually predominated over time [59] and glycosylated antigens cannot be produced in bacteria. A large competing population of bacteria importantly diminishes the chances of colonization and subsequent induction of a vigorous immune response. Oral delivery of live attenuated recombinant viruses such as adenoviruses, poxviruses, influenza, herpes viruses, and polioviruses encoding specific antigens has been also tested in several oral vaccine studies. While these viral vectors showed promising results, pre-existing immunity to these viruses may prevent their ability to deliver desired antigens. Moreover, live vaccines, in theory, carry a risk of virulence, and occasionally, they have other vaccine-associated effects. For example, oral polio vaccine contains a live attenuated poliovirus, which can infect the gastrointestinal tract and, subsequently, generate adequate immune protection into the host. The use of this vaccine was discontinued in the United States because of rare cases of vaccine-associated paralytic polio [60]. Today, it was replaced by inactivated poliovirus vaccine. Another live vaccine, RotaShield (Wyeth Ayerst) was also recalled owing to vaccine-related intussusceptions [61].

Despite considerable effort, oral immunization is still limited by several issues that are specific to this route. Typically, the doses that are required to elicit an immune response through the oral route are substantially higher (by up to 100-fold) than those that are required when using injection. This raises the crucial issue of the cost of immunization. Furthermore, oral immunization with non-living vaccines requires the use of carriers and adjuvants, and the safety of exposing the sensitive gastrointestinal tract to these compounds remains to be carefully studied. A completely different solution to this issue has been offered by transgenic plants which can be used as bioreactors for the production of mucosal delivery of protective antigens. This technology shows great promise to simplify and decrease the cost of vaccine delivery. This approach has yielded encouraging results in animals after oral administration of plant-based rotavirus VP6 which induces antigen-specific IgAs, IgGs and passive protection in mice [62]. The same type of approach has also been described in humans after oral administration of recombinant LT-B in transgenic corn [63]. In this study, 78% of volunteers developed rises in both serum IgG anti-LT and numbers of specific antibody secreting cells after vaccination and 44% of volunteers also developed stool IgA.

Furthermore, strong adjuvants such as bacterial enterotoxins (cholera toxin (CT); heat-labile *E. coli* enterotoxin (LT)) have also been successfully used for oral immunization in mice. CT is known to be endocytosed and transported by M cells and delivered into the subepithelial dome region, thereby stimulating immature DCs [64]. Recently, a rice-based vaccine that expressed cholera toxin B subunit is an effective long-term cold chain-free oral vaccine that induces CTB-specific SIgA-mediated longstanding protection against *V. cholerae* – or LT – ETEC-induced diarrhea [65]. However, toxicity of these enterotoxins limits their applications and that's probably why no studies in humans have been reported.

The ability of lectins to activate the immune system may be also exploited for oral administration. Lectins must enhance intestinal absorption by attaching to M cells in PP. For example, *Ulex europaeus 1* (UEA1), a lectin specific for α-L-fucose residues, binds almost exclusively to the surface of mouse Peyer's patch M cells [66]. Plant lectins have been demonstrated to be strong mucosal immunogens, stimulating systemic and mucosal antibody responses after oral or intranasal delivery. Mistletoe lectin-1, tomato lectin, *Phaseolus vulgaris*, wheat germ agglutinin (WGA), and UEA1 stimulated the production of specific serum IgG and IgA antibody after three oral doses in a mouse model [67,68]. However, M cell glycosylation patterns are not common to all species, and it remains to be seen whether it can be used to effectively target human M cells [69]. Human M cells have proven to be largely anonymous, as it has been difficult to isolate enough of such cells for further characterization and functional evaluation. Specific receptor requirements for human M cells and its specific targeting remains an important challenge. M cell targeting in mouse may be achieved using M cell-specific lectins, toxins, or nanoparticles. Recently, the first specific monoclonal antibody (mAb NKM 16-2-4) targeting murine M cells; has been described [70]. They used this antibody as a carrier for M cell-targeted mucosal vaccine. Oral administration of tetanus toxoid or botulinum toxoid conjugated with NKM 16-2-4, together with the mucosal adjuvant cholera toxin, is able to induce high-level of antigen-specific serum IgG and mucosal IgA responses.

IgA also adhere to M cell apical membranes by reverse transcytosis, regardless of its antigen specificity [71]. This was first observed in suckling rabbits as a local accumulation of milk SIgA on M cells of Peyer's patches [72]. This observation suggested it might be possible to exploit IgA for M cell targeting. However, this would require that the foreign epitope be inserted without affecting the molecular folding of secretory component (SC) or the assembly and the function of SIgA. Also, the site of insertion must be surface-exposed, and the inserted epitope must be immunogenic. *Shigella flexneri* invasin B epitope has been inserted into SC, which, following reassociation with IgA was delivered orally to mice. This immunization elicits both a systemic and mucosal response against the inserted epitope, thereby suggesting that recombinant SC IgA complexes could serve as a mucosal vaccine delivery system [73].

Although oral vaccines have several attractive features, the limited numbers of approved oral vaccines attest to the challenges associated with mucosal vaccine design. Studies involving oral vaccine use have been limited due to several challenges, such as difficulties in the collection and processing of external secretions, a lack of standardized assays, the induction of tolerance, the stability of antigens in the harsh conditions of the GI tract, and the antigen–microbial interactions that are continuously occurring in the large intestine [74]. Five of six approved vaccines (Table 1) are live attenuated vaccines, which are administered via the same route as the natural infection route of the corresponding pathogen and closely mimic the original pathogen. Although live attenuated vaccines are generally very effective, they may induce more adverse reactions than subunit vaccines. Additionally, in contrast to inactivated vaccines, live attenuated vaccines carry the risk of reassortment with wild type pathogens, thereby regaining their pathogenicity and not all pathogens can be attenuated for live vaccines. Subunit vaccines, however, have the disadvantage of being less immunogenic and therefore require potent delivery systems [75].

2.2. Other oral routes (Table 4)

The potential of the oral mucosa route, including buccal (the cheek lining), sublingual (underside of the tongue), and gingival mucosa, for administering vaccines is gaining increased interest because of recent studies indicating that this route may favour induction of broadly disseminated mucosal and systemic immune responses [76,77] and may offer a safer alternative to nasal delivery vaccines. Moreover, vaccine formulations administered by this route are directly bring in the bloodstream without passing through the intestine or liver and thereby are not subject to the degradation or the tolerance associated with gastrointestinal administration. Sublingual administration of a variety of soluble, as well as particulate antigens, including live and killed bacteria and viruses, can evoke a broad spectrum of immune responses in mucosal and extra-mucosal tissues, ranging from secretory and systemic antibody responses to mucosal and systemic cytotoxic T-cell responses [78]. Nowadays, sublingual route is only use in human for allergen-specific immunotherapies [79] and there is no background about immune responses to vaccines by this route except in mice. Therefore, sublingual mouse mucosa has been studied and it has been shown that within 2 h after sublingual administration with cholera toxin (CT), significantly higher numbers of MHC class II + cells accumulated in the sublingual mucosa. In reconstitution experiments using OVA-specific CD4+ or CD8+ T cells, sublingual vaccination elicited strong Ag-specific T cell proliferation mainly in cervical lymph nodes (CLN) [80]. Cuburu et al. [77] have recently reported in mice that sublingual administration of human papillomavirus virus-like particles (VLPs) with or without the CT adjuvant induced HPV-neutralizing Abs in serum, virus-specific Abs in genital tissues and conferred protection against genital challenge with HPV pseudovirions. Those findings are very promising for the development of vaccines against sexually transmitted diseases (STD). Moreover, it was recently reported in mice that sublingual immunization with HIV-1 gp41 and a reverse transcriptase polypeptide coupled to the cholera toxin B subunit (CTB) induced gp41-specific IgA antibodies and antibody-secreting cells, as well as reverse transcriptase-specific CD8 T cells in the genital mucosa [81]. Furthermore, a recombinant Ad5-based HIV vaccine vector expressing HIV-Gag can induce antigen specific CTL responses in both the systemic and mucosal compartments following sublingual vaccination that are; at minimum, equivalent to responses induced following oral gavage vaccination. Those results illustrate the ability for Ad vectors expressing a TLR agonist to not only induce heightened innate immune responses, but also to circumvent pre-existing Ad5 immunity and improve the antigen specific T-cell responses in a sublingual model of vaccine delivery [82]. These findings underscore the potential of the sublingual mucosa to serve as a potent route of vaccine delivery for inducing protective genital antibody and cellular immune responses able to limit the genital transmission of HIV-1 or other STD in a prime-boost strategy. However, there are differences in the anatomy of sublingual tissue and organization between mice and humans (keratinized and non-keratinized, respectively); so, we have to keep in mind that this approach could not be translated to humans.

2.3. Nasal delivery of vaccines (Table 3)

NALT lies in the roof of the nasopharynx in large mammals, at the caudal end of the pharyngeal septum. In rodents, the tissues are paired and lie on either side of dorsal surface of the hard palate. The NALT is easily overlooked, because macroscopically it frequently appears similar to the surrounding nasopharyngeal epithelium. In some species, such as sheep and cattle, the tissue is characterized by furrowing, especially in young animals, whereas in other species it is only recognizable grossly through the underlying follicles giving the surface a granular appearance. This organ is the only site in the respiratory system of sheep and primates in which FAE takes up particulate antigens for presentation to underlying lymphocytes. Like GALT in all mammalian species, rodent NALT has a smooth surface of dome epithelium with M cells, while human palatine tonsils and adenoids have deep and branched antigen-retaining

Table 3
Nasal delivery of vaccines.

Delivery strategies	Examples	Responses	Model	Reference
Chitosan	Influenza, pertussis, and diphtheria	– Serum IgG responses similar to and secretory IgA levels ↗	Animals/Human (influenza)	[98]
	N-trimethyl chitosan (TMC) nanoparticles	– Protection against the appropriate challenge	Mouse	[109]
	The nasal diphtheria vaccine with chitosan	– Systemic T cell responses ↗ – Antigen-specific IFN-gamma production ↗ – Th2-type responses ↗ – Protective levels of toxin-neutralizing antibodies ↗	Human	[99]
	Aminated gelatin microspheres (AGMS)	– Nasal absorption of insulin ↗	Rat	[110]
Cyclodextrins	Dimethyl-beta-cyclodextrin as adjuvants for nasally applied DT and TT	– Specific serum IgG titres ↗	Mouse	[85]
Liposomes	Liposomes incorporated insulin and coated with chitosan and carbapol	– Plasma glucose level up to 2 days ↘	Rat	[101]
Nanoparticles	Coated poly(anhydride) nanoparticles with either flagellin from *Salmonella enteritidis* or mannosamine	– Serum titers of IgG2a and IgG1 ↗ – TH1 and Th2 response ↗	Mouse	[111]
	Nanoencapsulated reporter plasmid (encoding β-galactosidase protein)	– Antibody levels ↗	Mouse	[112]
Modified vaccinia virus Ankara (MVA) vector	MVA expressing HIV-1 Env IIIB Ag	– Immune response to the HIV Ag ↗ – Mucosal CD8(+) T cell response in genital tissue and draining lymph nodes ↗ – Mucosal IgA and IgG Abs in vaginal washings ↗ – Specific secretion of beta-chemokines ↗	Mouse	[108]

↗: Increase. ↘: Decrease.

crypts with a reticular epithelium containing M cells [83]. This difference probably explains that germinal centres develop shortly after birth in human NALT as in the heavily microbe-exposed GALT, whereas rodent NALT requires infection or a danger signal. This is an intriguing species difference when it comes to induction of B-cell diversity and memory by vaccination via the nasal route [84]. Intranasal vaccines delivered into the nostrils are an attractive mode of immunization and the nasal mucosa is a practical site for vaccine administration because of the absence of acidity, lack of abundant secreted enzymes and small mucosal surface area that result in a low dose requirement of antigen. Furthermore, the nose is easily accessible, is highly vascularized, can be used for the easy immunization of large population groups. It is well established that nasally administered vaccines can induce both mucosal and systemic immune-responses, especially if the vaccine is based on attenuated live cells or if the antigen is adjuvanted by the use of an immunostimulator or a delivery system. This data were confirmed after nasal immunization of humans against diphtheria, tetanus [85], influenza [86] and infection with *Streptococcus mutans* [87]. In addition, it has been described in animal studies, potent responses in the respiratory and genital tracts could be induced by intranasal immunization, as a consequence of the common mucosal immune system [88,89]. The literature contains various examples of how intranasal vaccination can lead to a response at a distant mucosal site. For example, studies with cholera toxin B subunits showed that nasal mucosal immunization produced an exceptionally strong immune response in the respiratory and genito-vaginal tracts [90]. The optimized formulation and delivery system should be able to improve stability and retention of antigen in the nasal mucosa, increase uptake of antigen by the NALT and also potentially impart an adjuvant effect to the antigen without any toxic effect.

It is clear that the performance of a nasal vaccine can be greatly influenced by the physical nature of the antigen and the chosen delivery system. As shown in Table 3, different nasal vaccine systems in human and animal have been described either using live or attenuated whole cells, split cells, proteins or polysaccharides and with and without various adjuvants and delivery systems [91]. Different systems were shown to be efficient not only in mice but also in humans. Some of them have reached Phase I/II or Phase III clinical evaluation or are marketed. For example, FluMist™ (MedImmune Vaccines Inc.), a live influenza-virus vaccine, has already been approved by the Food and Drug Administration (United States) for intranasal administration. This vaccine has been studied in adults and children as young as 15 months for efficacy, immunogenicity, and safety. Among children the vaccine is safe, well tolerated, and up to 93% effective against culture-confirmed influenza [92]. Another study showed the safety and immunogenicity of a nasally administered vaccine comprising three monovalent inactivated influenza antigens associated with outer membrane proteins of *Neisseria meningitidis* (Proteosome) in normal, healthy adults. A positive and statistically significant antibody response was observed, in serum and in nasal secretions, to increasing dose for all three antigens [93]. Another live attenuated intranasal influenza vaccine, Nasalflu (Berna Biotech AG), was introduced in Switzerland in 2000. Although no serious adverse events were reported in prelicensure trials, during post licensure surveillance, Mutsch et al. showed the vaccine associated incidence of Bell's palsy, which was thought to originate from the use of *E. coli* LT as an adjuvant [94]. Efforts to develop other nasal vaccines, however, have continued to make progress.

Another approach to nasal vaccination involves the use of chitosan as a nasal vaccine delivery system. Chitosan is a linear polysaccharide biopolymer produced by deacetylation of chitin. Due to its biodegradability, biocompatibility and bioadhesive properties associated to a low toxicity, chitosan is widely used in intranasal formulations in animal studies. It is believed that it interacts with protein kinase C system and opens the tight junctions between epithelial cells [95], enhancing the transport of the drug across the membrane. Furthermore, chitosan may also protects the drug from enzymatic metabolism and sustains drug release, prolonging its effect [96]. But the normal mucociliary clearance time in healthy human's nose is about 20 min [97]. So for this strategy may be applicable to humans, it is important that the antigen should not release to a large extent before the antigen-loaded nanoparticles pass the nasal mucosal barrier during their residence in the nasal cavity. Chitosan has been tested with three different vaccines, namely for influenza, pertussis, and diphtheria in various animal models and in humans [98]. McNeela data

showed in humans that formulation of the nasal diphtheria vaccine with chitosan significantly augmented Th2-type responses, which correlated with protective levels of toxin-neutralizing antibodies in intranasally boosted individuals [99]. Chitosan has also been shown to improve the adjuvanticity of secondary immunomodulators like MDP, known to elicit cell-mediated immunity and CT subunit B, a potent mucosal adjuvant that induces strong humoral responses following nasally administered recombinant *Helicobacter pylori* urease in mice [100].

Liposomes, phospholipids vesicles composed by lipid bilayers enclosing one or more aqueous compartments and wherein drugs and other substances can be included, have been found to enhance nasal absorption of wide spectrum of antigens [101]. The adjuvanticity of liposomes is ascribed to their ability to accommodate multiple copies of antigenic epitopes, hence preferentially endocytosed by macrophages as well as their ability to protect the antigen within the biological environment and effects on the intracellular processing of antigen following uptake. Although this approach seems to be promising, it has some limitations in terms of sensitivity to host enzymes, instability on storage, and high cost of manufacture, which have encouraged the development of modified lipid-based vaccine delivery systems, such as ISCOMs, virosomes, proteosomes, proteoliposomes, and cochleates. Only virosomes and proteasomes have been tested in humans. Intranasal evaluation of virosomal influenza vaccines in a Phase I study showed that it was necessary to co-adjuvant with LT to obtain a humoral response comparable to that with parenteral vaccination [102]. Initial clinical study in healthy volunteers has shown intranasally administered proteosome-based influenza and shigella vaccines to be efficacious and well-tolerated [103]. These other modified lipid-based vaccine delivery systems offer a possible efficacious vaccine delivery system; however, there are reservations regarding toxicity and must be evaluated in humans.

In the last years, many efforts have been directed toward the enhancement of vaccine delivery by using polymeric nanoparticles as adjuvants or drug carriers for nasal immunization, in which the active substance is dissolved, entrapped, encapsulated, adsorbed or chemically attached [104]. Nanoparticles are probably taken up by M-cells in the nasal associated lymphoid tissue and, therefore, transported into the lymphatic system and blood stream [104]. Nanoparticles may offer several advantages due to their small size, but only the smallest nanoparticles penetrate the mucosal membrane by paracellular route and in a limited quantity because the size of the tight junctions. The efficacy of these nanoparticles to increase systemic and mucosal immune responses after intranasal administration is of interest to many groups. A lot of different studies (conducted largely in mice) indicated variable immune responses. In some cases, the serum IgG responses were increased when an antigen was administered in or on a microparticle, however, other cases, a decreased or unchanged response was found. Importantly, only in a few instances were mucosal IgA responses increased through an encapsulation strategy as compared to the administered free antigen [105].

Modified vaccinia virus Ankara (MVA) vector has also be used as mucosal vaccine. It is a highly attenuated strain of vaccinia virus that was developed towards the end of the campaign for the eradication of smallpox by Pr. A. Mayr in Germany. Recombinants based on the MVA vector were effective in inducing protective responses against different respiratory viruses, such as influenza and respiratory syncytial virus, following immunization via mucosal routes [106,107]. Gherardi et al. shown that intranasally inoculation of MVA expressing HIV-1 Env IIIB Ag induced a significant immune response to the HIV Ag [108].

To be effective following intranasal delivery, the formulation should ensure stability of the antigen, retain long enough time for the antigen to interact with the lymphatic system, stimulate both the innate and cellular systems with or without the use of safe efficacious adjuvants, by targeting specific parts of the immune cells, and provide long-term immunity against the pathogen. The critical issue in the selection of an adjuvant and/or delivery system for a viable human nasal vaccine is that it not only induces the required immune responses, but most of all also maintains the integrity and the various functions of the whole nose avoiding reactogenicity. Hence, it is of great importance that one considers carefully the choice of an absorption enhancer for a nasally delivered drug that is not readily absorbed especially in terms of potential nasal and systemic toxicity. Intranasal vaccines delivered into the nostrils are an attractive mode of immunization but we have to keep in mind that there are risks of passing into the brain through olfactory nerves and could induce important side effects.

2.4. Vaginal delivery of vaccines (Table 4)

Many pathogens are transmitted sexually through the genital tract (e.g., human immunodeficiency virus (HIV), human papillomavirus (HPV), *Chlamydia*, *Neisseria gonorrhoea* and herpes simplex virus). Protective mucosal immune response against sexually transmitted infections (STIs) should be triggered in vaginal mucosa.

Compared with the intestinal mucosa, mucosal surfaces of the female genital tracts are separated from the outside world by distinct epithelial cell layers and types of mucus that are inhabited by unique microflora and use distinct innate and adaptive effector mechanisms. In the genital mucosa, various innate immune cells provide defence against invading pathogens and epithelial cells are active participant in mucosal defence. There function as sensors that detect dangerous microbial component through PRRs which are essential for the generation of effective adaptive immune response to pathogens. Specific immune cells are also present in genital mucosa that provide immune protection as intraepithelial γδ T cells, macrophages, Langerhans cells (LCs) and submucosal DCs which are present in type II epithelia of the vaginal canals. In the uterus, specialized natural killer (NK) cells and regulatory T cells contribute to antiviral defences. After infection, neutrophils, monocytes, plasmacytoid DCs (pDCs) and NK cells are mobilized to the vaginal tissue. At later time point, antigen-specific T and B cells enter the tissue to provide pathogen-specific immune defence. In the steady state, LCs in the epithelium and DCs in the submucosa are highly phagocytic and express several PRRs that can recognize a wide array of microorganisms. After pathogen recognition through PRRs, DCs and LCs undergo a maturation program and migrate to the draining lymph nodes to prime naive T and B cells.

An important characteristic of the genital mucosal adaptive immune response is the local production and secretion of dimeric or SIgA antibodies that are resistant to degradation in the protease-rich external environments of mucosal surfaces. IgG as well as SIgA, could play a significant role in blocking infection by sexually transmitted pathogens at this site. Large numbers of IgG-secreting plasma cells are present in the female genital tracts of macaques and humans [109], and high concentrations of IgG and IgA have been measured in human cervical and vaginal secretions [110,111]. However, the vaginal mucosa is characterized by the absence of histologically demonstrable MALT. Priming occurs exclusively in the draining lymph nodes. The human vaginal canal is drained by several lymph nodes, including the common iliac, interiliac, external iliac and inguinal femoral lymph nodes.

For viruses that undergo rapid mutation, such as HIV-1, it has been shown that a large dose of vaginally administered gp120-specific monoclonal antibodies prevented vaginal SHIV transmission in macaques [112]. Many vaccine approaches have been tested but to date, none of these has proven to be successful in preventing HIV-1 infection in humans, despite inducing

Table 4
Other vaccine mucosal routes.

Other routes	Characteristics	Examples	Model	Reference
Sublingual	Antigen diffuses into the connective tissue and enters the venous circulation	HIV-1 gp41 and a reverse transcriptase polypeptide coupled to the cholera toxin B subunit	Mouse	[81]
		A recombinant adenovirus [E1-] Ad5-based HIV vaccine vector expressing HIV-Gag	Mouse	[82]
Aerosol	Simplified logistics, reduced cost per dose delivered, and greater safety/permits use of greater antigen volumes, and allows easier monitoring of results/achieve mass and rapid immunization	Live, attenuated EZ measles	Human	[122]
Ocular	Generate ocular mucosal immunity	Heat-killed HSV/HSV-based subunit vaccines	Mouse	[124]
		Recombinant HSV-2 glycoproteins B and D in MF59 emulsion	Rabbit	[125]
Vaginal	Local production and secretion of dimeric or SIgA antibodies/absence of histologically demonstrable MALT/drained by several lymph nodes	Polyanion PRO 2000 coated with HIV-1 envelope glycoprotein	Mouse/Rabbit	[113]
		Rheologically structured vehicle coated with an HIV-1 envelope glycoprotein	Rabbit	[114]
		Vaginal DNA vaccination using a needle-free jet injector	Rabbit	[115]
		CpG ODN in combination with glycoprotein D of herpes simplex virus type 2	Mouse	[119]

robust levels of antibody or cell-mediated immunity. Recently, vaginal immunization of polyanion PRO 2000 coated with HIV-1 envelope glycoprotein resulted in significantly increased titres of Env-specific mucosal IgA and IgG in mice and rabbits, compared to Env alone, revealing modest but significant PRO 2000 mucosal adjuvant activity for [113]. The polyanion PRO 2000 is safe for human vaginal application, and thus may represent a potential formulating agent for vaginal delivery of experimental vaccine immunogens. Rheologically structured vehicle (RSV) gels may be also developed as delivery systems for vaginal mucosal vaccination with an HIV-1 envelope glycoprotein. Indeed, vaginal administration to rabbits induced both systemic IgG and mucosal IgG/IgA in genital tract secretions [114]. RSVs could be a viable delivery modality for vaginal immunization but need to be evaluated in humans.

Vaginal DNA vaccination using a needle-free jet injector is a promising approach for the prevention and treatment of mucosal infectious diseases. Mucosal gene transfection efficiency of a non-needle jet injector for daily insulin injection at the skin and vagina levels could be an attractive approach. Intravaginal immunization in rabbits promotes vaginal IgA secretion and IFN-gamma mRNA expression in blood lymphocytes, to a degree significantly higher than needle-syringe injection [115]. Jet injection has been tested subcutaneously in several human clinical trials [116] and is already produced commercially for daily injection of insulin and growth hormone. However, no needle-free jet injection into the vaginal mucosa has been previously reported into human trials.

The estradiol-induced mucus barrier may be a novel approach to prevent exposure to antigens delivered intravaginally. Estradiol, which induces an estrus-like state, has been shown to prevented CD8(+) T cell priming during intravaginal immunization of mice [117]. Furthermore, it has been demonstrate that estradiol prevented antigen loading of vaginal antigen presenting cells (APCs) after intravaginal immunization of mice [118].

CpG-containing oligodeoxynucleotide could be used for induction of chemokine responses in the genital tract mucosa and also as a vaginal adjuvant in combination with an antigen for induction of antigen-specific immune responses. A single intravaginal administration of CpG ODN in combination with glycoprotein D of herpes simplex virus type 2 in mice stimulates a rapid and potent chemokine response both in the vagina and/or the genital lymph nodes [119]. These findings highlight the value of CpG ODN as a potent mucosal adjuvant for the induction of protective Th1-tilted immune responses against genital herpes and justify further investigation of the use of CpG ODN for the development of mucosal vaccines against other sexually transmitted pathogens, such as HIV.

Despite several advantages, as compared to systemic injections, the delivery of vaccines by genitals routes has not been shown to be very practical in human trials. Indeed, it is cumbersome to administer a mucosal vaccine through the genital tract, as the immunological features of the female reproductive tract, in particular, alter dramatically in response to hormonal fluctuations during the menstrual cycle [110]. In addition, female genital tracts lack inductive mucosal sites analogous to intestinal Peyer's patches. Recently, a study compared the protective activity resulting from immunization of mice via intranasal, intravaginal or subcutaneous routes with an adjuvanted HPV type 16 E7 polypeptide vaccine. They demonstrate that only subcutaneous immunization induced complete regression of already established genital tumors [120].

2.5. Other vaccine mucosal routes (Table 4)

Additional mucosal routes, including pulmonary and ocular administration of vaccines, have also been attempted in several cases.

Aerosol vaccination, which best follows the natural route of many infections, aims to deliver vaccine at various levels of the bronchial tree, including the alveoli and may first leads to development of immunity at the portal of entry, and may also induce a more generalized defence. Compared with injections, the aerosol method has simplified logistics, reduced cost per dose delivered, and greater safety. Indeed, the recommended optimal way of introducing an aerosol vaccine is nasal breathing, which is more suitable for geriatric and pediatric populations, permits use of greater antigen volumes, and allows easier monitoring of results. It was shown in human that aerosol immunization is at least equivalent to injections of measles, rubella and mumps (MMR) vaccines in eliciting high levels of protection for at least 1 year following revaccination [121]. Bellanti et al. immunized Mexican school children against measles and they shown that serum and nasal IgG and IgA antibody responses were stimulated following immunization with live, attenuated Edmonston-Zagreb (EZ) measles vaccine administered either by aerosol or subcutaneous routes but these responses were significantly greater by the aerosol compared to the subcutaneous route [122]. Although some basic information are still lacking, aerosol immunization has already been used successfully in large populations and has therefore passed the phase of initial feasibility evaluation.

Ocular immunization has been attempted in rabbits against infection with herpes simplex virus (HSV). This was motivated by the strong need to generate ocular mucosal immunity to HSV,

which commonly infects the eye in addition to other sites. The combined efficacy of heat-killed herpes vaccine and acyclovir inoculated by intraocular route generated effective mucosal immunity to HSV and permitted to protect all animals [123,124].

Another study shown ocular vaccination provided better protection than systemic vaccination against eye disease following ocular HSV-1 infection. Indeed, periocular vaccination with recombinant HSV-2 glycoproteins B and D in MF59 emulsion provided significant protection against conjunctivitis and iritis, while ocular vaccination with a non neurovirulent strain of HSV-1 provided significant protection against conjunctivitis, iritis, epithelial keratitis, and corneal clouding. Systemic vaccination with either HSV-1 KOS or gB2/gD2 in MF59 did not provide significant protection against any of four parameters [125]. However, ocular routes have been less well studied as generalized methods for immunization than have other mucosal routes, but recent developments in cellular and molecular immunology of the ocular mucosal immune system may help in the design of more effective and optimal immunization strategies against ocular pathogens.

As the vaginal mucosa is characterized by the absence of histologically demonstrable MALT, it should be interesting to deliver antigens in the anorectal canal where they could be processed by the local MALT, which underlies the type I epithelium, and by the inguinal lymph node, which drains the lower rectum and anus. Live vaccine vectors and adjuvants that cannot be used orally or nasally might be safe and effective if administered by the rectal route, however, this possibility warrants testing in human studies and rectal route is limited by acceptability and immunogenicity issues.

3. Conclusions

Currently, there are few mucosal vaccines in use, due to the poor efficiency of new strategies. A common theme that has emerged is that the natural immunity that develops following infection with mucosal transmitted diseases provides minimal protection against secondary challenges with a heterologous pathogen. Thus, a vaccine may need to elicit a different type of immune response altogether to achieve protection in the immunized hosts.

However, the ineffective protection afforded by traditional injected vaccines against a large number of pathogens (HIV, malaria, tuberculosis, etc.) has led to active research and development of alternative routes of immunization, such as the mucosal route.

The development of safe and effective mucosal adjuvants remains a particular priority for non-replicating mucosal vaccines. These would offer the potential to induce vaccine specific responses at the mucosal portals of pathogen entry. So it is necessary to develop non-toxic and effective adjuvants which could permit the use of low immunogenic non-replicating antigens when administered by mucosal routes. Moreover, the use of an adjuvant competent for mucosal administration could permit targeting of mucosal immune cells and generate the desired immune response. A comparison of the advantages and limitations of various methods of mucosal immunization makes it evident that there is no one superior method but there is a drawback that they share: the fact that there is a lack of control in terms of dose that is delivered (as opposed to systemic administered) to each individual and it is an important problem during the vaccines studies.

The choice of any given route of mucosal vaccination and the selection of appropriate adjuvants and formulations will affect vaccine design, process and manufacturing issues. Much work remains to be done, but current research continues to clarify the concepts and provide the tools that are needed to exploit the full potential of mucosal vaccines. However, those researches are by a majority done on animal models and it is very important to keep in mind that often those models cannot be extrapolate to human due to the very significant differences in anatomy, physiology and immunogenicity. The NALT, sublingual and genital epithelia of rodents have significant differences to that of humans. For example, the vaginal mucosa of rodents is a keratinized tissue, so it is not a good model for human (vaginal mucosa with nonkeratinized surface) and will tend to be less permeable and more resistant to damage [126]. TLR expression patterns are different; rodents constitutively make dimeric IgA irrespective of the route of immunization, while humans make mIgA and dIgA. These differences are critical in determining the value of different experimental data and even if sometime mucosal vaccinations give interesting results in animals, it requires verification before they can be considered as promising. Indeed it is often seen that approaches that work well in rodent species fail to work in non-human primates or humans. New mucosal vaccines should be tested in human subjects, but must take into account the genetic background of the host, the intestinal microflora diversity and diet, all parameters which could influence the vaccine safety and effectiveness. Indeed, some mucosal vaccines, such as oral polio, cholera or typhoid vaccines are less effective in developing nation than industrialized countries. To explore the impact of environmental factors in relation to mucosal responses to vaccines administered by different routes, studies should be conducted with licensed vaccines; both killed and live bacterial and viral vaccines (e.g., OPV and IPV). Use of animal models could be helpful in exploring the influence of these factors on mucosal immune responsiveness to antigens and adjuvants administered by these different routes.

The antigens and adjuvants vectorization is a very interesting field for mucosal delivery vaccines, then the formulation of antigens in various particulate delivery systems for mucosal administration may be advantageous in the following ways: (i) protection of the antigen from mucosal enzymes; (ii) facilitating the preferential uptake of encapsulated antigen by specialized NALT/GALT/BALT M cells; (iii) sustained release of antigen to increase the presentation time of antigen to APC; (iv) possible increase in retention time at the site of administration by bioadhesion; (v) co-presentation of antigen and adjuvant to APCs, and (vi) induction of cell-mediated immune response by modifying presentation of antigen to APCs [127,128,49]. To conclude, rational antigen selection, adjuvants to angle protective immune responses, efficient vectors to target APCs and appropriate administration routes should permit to develop efficient mucosal vaccines.

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