Mucosal Delivery Routes for Optimal Immunization: Targeting Immunity to the Right Tissues

C. Czerkinsky and J. Holmgren

Abstract The mucosal immune system exhibits a high degree of anatomic compartmentalization related to the migratory patterns of lymphocytes activated at different mucosal sites. The selective localization of mucosal lymphocytes to specific tissues is governed by cellular "homing" and chemokine receptors in conjunction with tissue-specific addressins and epithelial cell-derived chemokines that are differentially expressed in "effector" tissues. The compartmentalization of mucosal immune responses imposes constraints on the selection of vaccine administration route. Traditional routes of mucosal immunization include oral and nasal routes. Other routes for inducing mucosal immunity include the rectal, vaginal, sublingual, and transcutaneous routes. Sublingual administration is a new approach that results in induction of mucosal and systemic T cell and antibody responses with an exceptionally broad dissemination to different mucosae, including the gastrointestinal and respiratory tracts, and the genital mucosa. Here, we discuss how sublingual and different routes of immunization can be used to generate immune responses in the desired mucosal tissue(s).

Contents

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1 Introduction

The gastrointestinal, respiratory and urogenital tracts, eye conjunctiva, inner ear, and ducts of all the exocrine glands are covered by mucous membranes endowed with powerful mechanical and chemical cleansing mechanisms that repel and degrade most foreign matter (Brandtzaeg and Pabst 2004; Ogra 1999). In addition, a highly specialized innate and adaptive mucosal immune system protects these surfaces, and thereby the body interior, against insults from the environment. In a healthy human adult, this local immune system is estimated to comprise nearly 80% of all lymphocytes, commensurate with the ca. 400 square meters of mucosal surfaces that it has to defend. These immune cells are accumulated in, or in transit between, various mucosa-associated lymphoid tissues (MALTs), which together form the largest mammalian lymphoid organ system. The MALT represents a highly compartmentalized immunological system that functions independently of the systemic immune apparatus. At variance with the systemic immune system, which normally functions in a sterile milieu and can thus afford to respond vigorously to invaders, the MALT guards organs that are replete with foreign matter. Only a limited proportion of these foreign antigens are derived from pathogens; most are commensals, food proteins, and other ingested or inhaled foreign materials. This means that upon encounter with this broad range of antigenic stimuli, the MALT must select appropriate effector mechanisms and regulate their intensity to avoid bystander tissue damage and immunological exhaustion.

There is a great need to develop vaccines against many bacterial and viral pathogens. The majority of microbial pathogens have a mucosal port of entry. Although parenteral vaccination can provide protection in some instances, a mucosal vaccination route is necessary in most cases. In addition, as compared to injectable vaccines, mucosal vaccines would be easier to administer, carry less risk of transmitting infections, and could simplify manufacturing, thereby increasing the potential for local vaccine production in developing countries (Holmgren and Czerkinsky 2005).

In the early days of mucosal immunology, it was thought (based on studies in mice) that immune responses initiated at one mucosal site would be widely disseminated to multiple mucosal tissues. Had this common mucosal immune system existed, it would have meant that immunization of humans by the oral route could be used effectively to induce immune responses not only in the gastrointestinal tract, but also in the airways and the urogenital tract. However, further work has shown that mucosal immune responses are highly compartmentalized, not only between separate mucosal organs (Ogra and Karzon 1969), but also between regions within the same mucosal organ, such as the gut (Holmgren and Czerkinsky 2005; Ogra and Karzon 1969).

In this review, we summarize the anatomical distribution of immune responses after mucosal immunization by different routes. Whenever possible, the information provided is based on findings in humans or non-human primates since studies in mice may give misleading information. Most of our knowledge is from oral or site-specific immunization in the gastrointestinal tract, but some information exists for vaccination by the nasal or vaginal routes. We will also discuss, in some detail, the newer sublingual vaccine delivery approach. When compared with other vaccination routes, the sublingual route offers the potential to produce strong and more broadly disseminated T cell and antibody responses in systemic and mucosal tissues.

2 Mucosal Vaccines: An Unmet Need

Most, perhaps 90%, of all infections are caused by pathogens which have a mucosal portal of entry. There is an urgent need for vaccines that induce effective and long-lasting immunity, especially during infancy and early childhood, against numerous respiratory and enteric pathogens. It is estimated that mucosal respiratory and gastrointestinal infections kill approximately five million children under age five in developing countries and cause more than ten billion disease episodes each year. These diseases negatively impact growth, cognitive function, and quality of life. Similarly, there is a great need for vaccines that can protect against human immunodeficiency virus (HIV) and other sexually transmitted infections that affect millions of adults and adolescents. These conditions have a tremendous negative impact on global health and overall economic development. To date, more than 30 injectable vaccines have been licensed for human use, compared with only a handful of mucosal vaccines. All of these mucosal vaccines are for oral use against enteric infections with the exception of two nasal cold-adapted attenuated influenza vaccines (Table 1).

A large number of pathogens cause or initiate infections in the gastrointestinal tract (e.g. *Helicobacter pylori*, *Vibrio cholerae*, enterotoxigenic *Escherichia coli* (ETEC), *Shigella* spp., *Salmonella* spp., *Clostridium difficile*, polioviruses, rotaviruses and noroviruses). Several other pathogens cause acute or chronic respiratory infections (group A streptococci, *Streptococcus pneumoniae, Haemophilus*

Table 1 Internationally licensed mucosal vaccines currently used in humans

Oral polio virus vaccines (OPV)
Oral live-attenuated typhoid vaccine (Vivotif™)
Oral inactivated B subunit-whole cell cholera vaccine[a] (Dukoral™)
Oral live-attenuated rotavirus vaccines (RotaTeq™ and ROTARIX™)
Nasal cold-adapted live-attenuated influenza vaccine[b] (FluMist™)

[a] Domestically licensed killed whole cell oral cholera vaccines are also used in Vietnam, India, and China

[b] A cold-adapted live-attenuated nasal influenza vaccine is also licensed in Russia since 1961

influenzae, Mycoplasma pneumoniae, influenza virus, respiratory syncytial virus, and *Mycobacterium tuberculosis)*. There are also a number of sexually transmitted mucosal pathogens (e.g. HIV, human papillomavirus, *Chlamydia*, *Neisseria gonorrhea* and herpes simplex virus). Collectively, infections caused by these agents represent an enormous challenge toward the development of vaccines which induce protective immunity by either preventing the infectious agent from attaching to and colonizing the mucosal epithelium (non-invasive bacteria) or from penetrating and replicating in the mucosa (viruses and invasive bacteria), or by blocking the binding of microbial toxins to epithelial and other target cells.

2.1 Considerations in Selecting a Mucosal or Parenteral Vaccine Delivery Route

It is highly probable that infection by and inter-person transmission of most mucosal pathogens can be effectively controlled by mucosal vaccines provided these vaccines are rationally designed and formulated to be administered through an appropriate route. However, the nature of the pathogen and of the target mucosal tissue will determine whether the vaccine should be given mucosally or parenterally to be efficacious (Fig. 1). A topical mucosal vaccination route seems to be critical for protection against non-invasive infections at mucosal surfaces and such infections involve pathogens that remain on the apical (luminal) side of mucosal epithelia, i. e. at sites (i) that are poorly accessible to antibodies transudating from blood, and (ii) where blood-derived monomeric IgG or IgA are insufficiently concentrated on the apical cell surface (due to the lack of receptor-mediated transport) or are unstable to function in the external mucosal environment. Cholera and ETEC are typical examples of infections in which vaccine-induced protection appears to be mediated mainly, if not exclusively, by locally produced secretory IgA (S-IgA) antibodies, and is associated with immunological memory.

On the other hand, when infection occurs at mucosal surfaces, such as those in the respiratory and urogenital tract, which are more permeable than the intestines to transudation by serum antibodies, a parenteral route of vaccination may be effective. The same may hold true for enteric infections where the pathogen is first

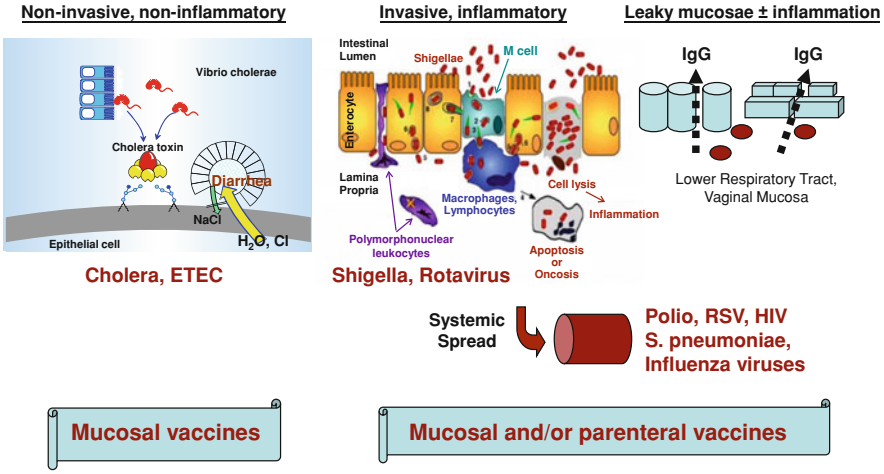

Fig. 1 Different types of mucosal infections and mucosal surfaces may call for different types of vaccines. Mucosal vaccination may be critical for protecting against non-invasive infections at mucosal surfaces that are normally impermeable to serum antibody transudation; V. cholerae and ETEC infections are examples of such infections, where vaccine-induced protection is mediated mainly, if not exclusively, by locally produced SIgA antibodies. Protection against more invasive pathogens or infections at mucosal surfaces that are permeable to transudation by serum antibodies, such as the lower respiratory tract or genital mucosae, may be achieved by either mucosal or parenteral vaccines

translocated across the epithelial barrier and then infects the basolateral side of the epithelium (as with *Shigellae*) or when the pathogen causes disease only after multiplying and producing inflammation in the submucosal tissues (as for *Campylobacter* and most *Salmonella* bacteria). Finally, parenteral vaccines are clearly efficacious for many viral and bacterial infections caused by pathogens that utilize a mucosal portal of entry, but then quickly enter the blood for systemic spread. Typical pathogens in this category for which effective injectable vaccines exist include *S. pneumoniae*, *H. influenzae*, *S. typhi*, poliovirus and influenza virus. It is notable that for the last three, mucosal vaccines are also available.

Taken together, and amid our gaps in knowledge of the mucosal immune system as well as our ability to measure its effector and memory arms, these considerations highlight the challenges to be met by vaccinologists when attempting to design, formulate, and deploy future mucosal vaccines.

3 Compartmentalization and Cell Migration in the Mucosal Immune System

The MALT comprises anatomically defined lymphoid microcompartments, which serve as the principal mucosal *inductive sites* where immune responses are

initiated (Brandtzaeg and Pabst 2004; Ishikawa et al. 1999; Kiyono and Fukuyama 2004). Examples of such inductive sites are the Peyer's patches in the small intestine (mainly in the ileum), the abundant lymphoid follicles in the appendix, colon and rectum; the mesenteric lymph nodes, the tonsils and adenoids at the entrance of the aerodigestive tract, and the many lymphoid follicles dispersed within the nasal mucosa and the bronchi of the respiratory tract (although the latter structures are less prominent in humans as compared to some animal species). The MALT also contains a diffuse accumulation of lymphoid cells in the parenchyma of mucosal organs and exocrine glands, which represent the mucosal *effector sites* where immune responses are expressed. Consistent with its high degree of compartmentalization, the MALT is populated by phenotypically and functionally distinct B cell, T cell, and accessory cell subpopulations, when compared with systemic lymphoid tissues; and it has also developed strong restrictions upon lymphoid cell recirculation between mucosal sites.

3.1 Induction of Mucosal Immune Responses

As extensively discussed elsewhere (Fahlen-Yrlid et al. 2009; Iwasaki 2007; Kraehenbuhl and Neutra 2000), antigens may either penetrate or be taken up in mucosal inductive sites through a variety of mechanisms. One such example is the gut where the presence of a mucosal lymphoid follicle influences the adjacent intestinal epithelium by inducing differentiation of M cells (Kraehenbuhl and Neutra 2000). The latter cells, which are most prominent over the Peyer's patches, have special properties for transporting antigens across the epithelial barrier (Jang et al. 2004). Recently, an additional mechanism has been proposed for the uptake of antigens at mucosal surfaces that can occur in the absence of an organized follicle-associated epithelium. This mechanism involves dendritic cells (DCs), which can protrude antigen-sampling dendrites across the intestinal epithelium and into the lumen (Rescigno and Di Sabatino 2009).

Irrespective of sampling mechanism, antigens taken up at a mucosal surface can be ferried to, or directly captured by professional antigen-presenting cells (APCs), and presented to conventional CD4+ and CD8+ $\alpha\beta$ T cells. Certain antigens may also be processed and presented directly by epithelial cells to neighbouring intraepithelial T cells, including T cells with limited repertoire diversity ($\gamma\delta$ T cells and NKT cells). With the majority of antigens, this results in the suppression of specific immunity or "oral tolerance" (Mowat 2003). However, an active immune response may also ensue, depending on the nature of the antigen, the type of APC involved, and the local microenvironment. In general, inflammatory conditions favor the development of productive immune responses, and these responses are triggered by pathogens harboring motifs that are sensed as "danger signals" after binding to Toll-like receptor (TLR) ligands on mucosal APC (Bilsborough and Viney 2004; Rakoff-Nahoum et al. 2004). The stimulation of the mucosal innate

immune system is an important reason why pathogens, live-attenuated bacterial or viral vaccines, and killed whole-cell bacterial vaccines induce an immune response rather than tolerance. Selected subunit vaccines may also induce strong immune response by possessing similar or functionally analogous motifs, but subunit vaccines typically need to be delivered with a pro-inflammatory adjuvant to stimulate a strong immune response. In most cases, a mucosal immune response appears to critically depend on appropriate antigen presentation by mucosal DCs, although a mucosal IgA response could be induced in DC-depleted animals when very high amounts of mucosal antigens were given (Fahlen-Yrlid et al. 2009).

3.2 Tissue-Specific Homing of Mucosal Lymphocytes

Sensitized mucosal immunocytes, both B and T cells and also IgA plasmablast precursors, leave the site of initial antigen encounter (e.g. Peyer's patch), transit through the lymph, enter the circulation, and then seed selected mucosal sites, preferentially the mucosa of origin, where they differentiate into memory or effector cells. Anatomic affinity of mucosal lymphoid cells appears to be largely determined through site-specific integrins ("homing receptors") and chemokine receptors and complementary tissue-specific endothelial cell adhesion molecules ("addressins") and chemokines which are expressed differentially in the various mucosal tissues (Berg et al. 1989). This explains why mucosal lymphocytes preferentially traffic to mucosal rather than peripheral organs and tissues. For instance, gut-homing IgA B cell precursors, their plasmablast progenitors, and memory T cells express $\alpha 4\beta 7$ integrin that specifically attaches to MadCAM-1, a tissue-specific addressin that is selectively expressed on the endothelium in the gastrointestinal tract (Kunkel and Butcher 2003).

Mucosal DCs in concert with neighboring epithelial cells play a critical role in this process by programming B and T lymphocytes to express tissue-specific homing receptors (Iwasaki 2007; Johansson-Lindbom et al. 2005; Mora et al. 2003, 2006; Rescigno and Di Sabatino 2009; Stagg et al. 2002). Likewise, chemokines produced by epithelial cells in the local microenvironment promote chemotaxis of immune cells with cognate chemokine receptors (Kunkel et al. 2003; Rescigno and Di Sabatino 2009). For instance, in the gastrointestinal tract, CCL28 selectively attracts IgA B cells and plasmablasts expressing the chemokine receptor CCR10, whereas CCL25 produced by small intestinal epithelia selectively attracts B and T cells expressing the CCR9 receptor from the blood into the small intestinal lamina propria (Kunkel et al. 2003). The tissue-specific imprinting of homing molecules and chemokine receptors on lymphocytes activated in mucosal inductive sites and the selective expression of addressins and chemokines in the target mucosal tissue explains the segregation of mucosal and systemic immune responses as well as the preferential dissemination of mucosal lymphocytes to privileged mucosal sites.

Table 2 Anatomic distribution of mucosal IgA antibody responses after immunization by different routes

	Immunization route					
	Nasal	Sub-lingual	Oral	Rectal	Vaginal	Trans-dermal
Upper respiratory tract	+++	+++	–	–	–	+++
Lower respiratory tract	+/+++[a]	+++	–	–	–	+++
Stomach	–	+[b]	+[b]	–	–	?
Small intestine	–	+++	+++	–	–	+
Colon	–	?	++	++	–	+
Rectum	–	?	±	+++	–	?
Reproductive tract	++	+++	–	–	++/+++[c]	?
Blood	+++	+++	+	+/±	+/±	+++

[a] Strong response only by aerosol administration
[b] Stronger response (+++) in *H. pylori*-infected individuals
[c] Strongest response when immunization is performed during the mid-follicular phase

4 Mucosal Effector Sites Associated with Vaccination Routes

The compartmentalization within the mucosal immune system places constraints on the choice of vaccination route for induction of immune responses at a desired site. Administration of antigens by rectal, vaginal, and more recently sublingual, routes has been explored but only for experimental purposes so far, and mainly for studying S-IgA antibody responses. In general, as summarized in Table 2, the strongest immune response is obtained at the site of initial vaccine exposure and in anatomically adjacent mucosal sites. However, a few notable exceptions have been found that may allow for more practical vaccine administration than would otherwise be possible, especially for infections in the urogenital tract. This has obvious implications for the development and deployment of mucosal vaccines.

4.1 Intestinal, Nasal, and Vaginal Vaccination

Traditional routes of mucosal immunization include the oral and nasal routes. If antigens with inherent immunogenicity are used either alone or co-administered with an effective adjuvant, oral immunization induces a substantial antibody response in mainly the small intestine (and then strongest in the proximal segment), in the ascending colon (Fig. 2), the stomach and in the mammary and salivary glands (Czerkinsky et al. 1991; Eriksson et al. 1998; Jertborn et al. 2001; Johansson et al. 2004; Quiding et al. 1991) (Table 2). Oral immunization is, however, relatively inefficient for evoking an IgA antibody response in the distal segments of the large intestine (Fig. 2), the tonsils, the lower airway mucosa, or

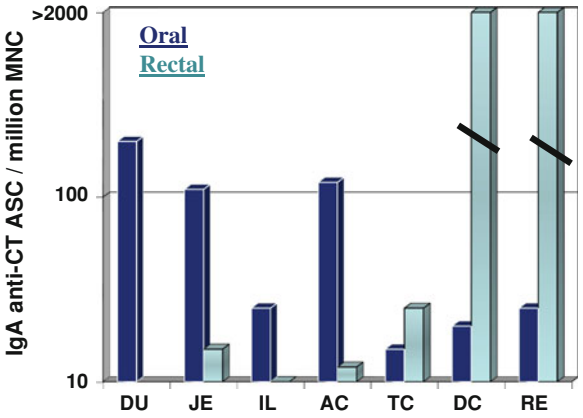

Fig. 2 Compartmentalization of mucosal immunity illustrated by the anatomic segmentation of the intestinal IgA immune response in macaques after oral and rectal immunizations with cholera toxin (two doses two weeks apart). Data are from Eriksson et al. (1998).

the reproductive tract mucosa (Eriksson et al. 1998; Kozlowski et al. 1997; Nardelli-Haefliger et al. 2003; Wassén et al. 1996). Conversely, rectal immunization evokes a strong local antibody response in the rectum, sigmoid colon, and descending colon (although weaker), but little, if any response, in the small intestine and ascending colon (Johansson et al. 2004) (Fig. 2) and distal reproductive tract (Kozlowski et al. 1997, 2002). Vaginal immunization, especially during the mid-follicular phase of the menstrual cycle, similarly induces strong local mucosal immune responses without producing notable distal immune responses (Johansson et al. 2001; Kozlowski et al. 1997, 2002; Wassén et al. 1996).

On the other hand, nasal or tonsillar immunization in humans produces IgA antibodies in the upper airway mucosa and regional nasal or salivary secretions without evoking an immune response in the gut (Quiding-Järbrink et al. 1995, 1997). However, for possible vaccination against HIV and other sexually transmitted infections, nasal immunization has been found to give rise to substantial IgA and IgG antibody responses in the human cervico-vaginal mucosae (Johansson et al. 2001; Kozlowski et al. 2002; Nardelli-Haefliger et al. 2003). The magnitude of the response achieved in the genital mucosa of women after intranasal immunization appears to be fully comparable to that seen when the vaccine is given by topical vaginal application (Johansson et al. 2001; Nardelli-Haefliger et al. 2003).

Apart from the anatomical differences in the dissemination of S-IgA antibody responses induced by oral and nasal immunization, respectively, the kinetics of the responses also appear to be markedly different. Several studies have shown that the intestinal immune response after oral immunization is rapid and relatively short-lived, although it is associated with long-lasting immunological memory. After oral cholera vaccination, data from extensive field trials in developing countries have shown that protection mediated by the acute intestinal IgA response appears to vanish after 6–9 months, but overall protection lasts for several years, consistent with the demonstration of mucosal immunological memory lasting for

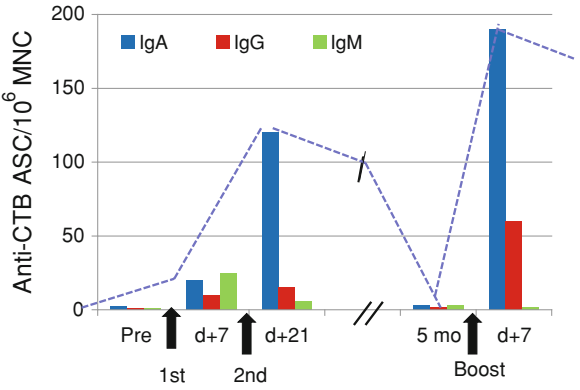

Fig. 3 Local intestinal antibody responses after primary and booster immunizations with oral cholera vaccine in Swedish human volunteers. Large numbers of IgA-antibody-secreting cells (ASC) (detected by ELISPOT on cells isolated from small intestinal biopsies) were observed after two oral immunizations and a third single dose given 5 months (or in another study 5 years) later evoked strong immunologic memory. Adapted from Quiding et al. (1991)

at least 5 years in Swedish volunteers after oral cholera vaccination (Jertborn et al. 1994). In experimental studies in humans, Quiding et al. (1991) examined the kinetics of the antibody-secreting cell (ASC) response to the cholera B subunit (CTB) in the small intestine after single and booster oral cholera vaccinations. They found that duodenal IgA ASC responses to CTB peaked 1 week after immunization and decreased markedly over a 5-month period, but these responses could be quickly recalled by a booster administration of vaccine (Fig. 3).

Rudin et al. (1998) compared the kinetics and organ distribution of the antibody response after nasal and oral vaccination. They immunized Swedish female volunteers nasally or orally with CTB and measured specific antibody in serum and in nasal and vaginal secretions at different times after immunization. Strong systemic antibody responses to CTB were induced by both routes of vaccination. Nasal vaccination strongly increased CTB-specific IgA in nasal secretions, whereas no significant nasal IgA response was seen after oral vaccination. A striking difference between nasal and oral vaccination was that the nasal route elicited an antibody response with a later onset but of much longer duration than the oral route in both serum and at the mucosal expression sites (Fig. 4).

4.2 Sublingual Vaccination

The above considerations have prompted efforts to identify alternative routes of vaccine delivery. In this respect, the potential of the sublingual ("under the tongue") route for administration of vaccines is gaining increased interest due to recent studies indicating that this route may in fact induce broadly disseminated mucosal and systemic immune responses. Over the past few years, we and others have shown that sublingual administration of a variety of soluble, as well as

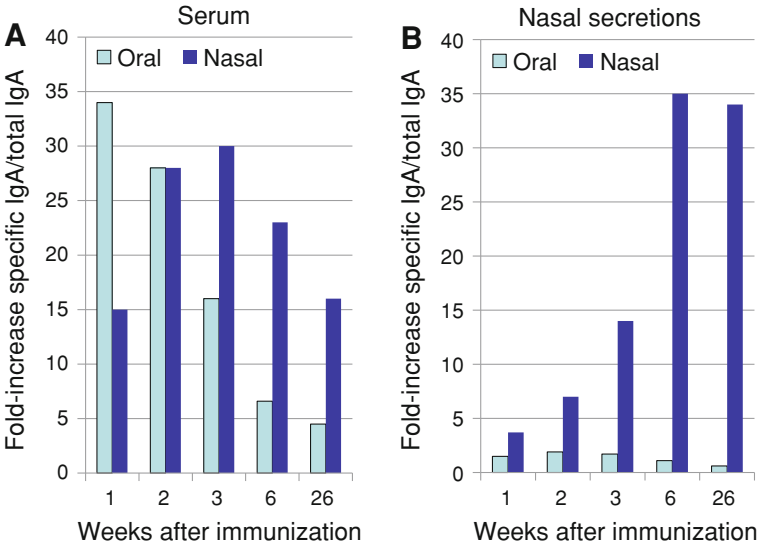

Fig. 4 Different kinetics of the mucosal and serum antibody response after mucosal immunizations by the oral as compared to the nasal routes. (B, C) Adapted from Rudin et al. (1998): The kinetics of both serum and mucosal antibody responses differ after oral (A) and nasal (B) immunization; nasal immunization gives rise to a less rapid but longer-lasting response than oral immunization.

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 lymphocyte (CTL) responses (Cuburu et al. 2007). Although only studied in animals so far, in all instances where this route of administration has been compared to the classical orogastric route, sublingually induced responses have been far more pronounced and required 10- to 50-fold lower amounts of antigen (Cuburu et al. 2007). Moreover, sublingual, but not oral administration of killed or live-attenuated influenza vaccine induced antiviral responses in the lungs of mice, and protected mice against lethal respiratory challenge with infectious virions (Song et al. 2008) (Fig. 5).

Importantly, antigens and adjuvants that have been administered sublingually are not redirected to the olfactory bulb epithelium; thus sublingual vaccines are less likely to have the same safety issues as nasal vaccines. More recently, we have documented that similar to nasal immunization, but at variance with orogastric immunization, sublingual administration of non-replicating antigens can also induce secretory antibody responses, and depending on the adjuvant used, CTL responses in the female reproductive tract (Cuburu et al. 2009). Another significant finding is that sublingual administration of a non-adjuvant vaccine consisting of human papillomavirus virus-like particles (VLPs) evoked virus-neutralizing antibody responses in serum and genital secretions, and provided protection against

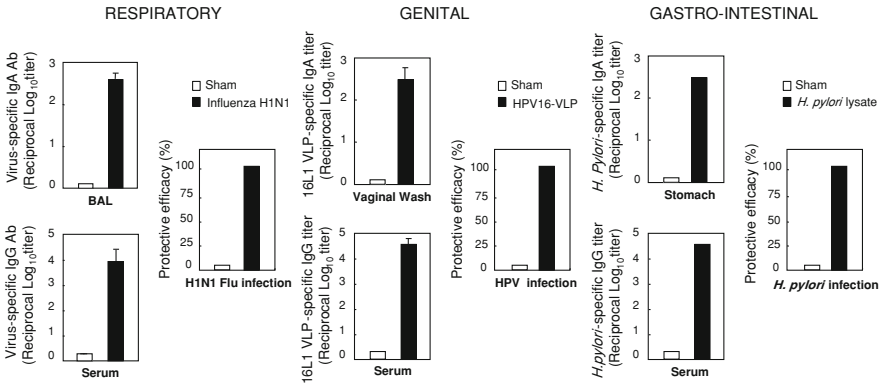

Fig. 5 Sublingual vaccination induces broadly disseminated protective immune responses. Systemic and mucosal antibody responses in mice after 2 consecutive immunizations with killed influenza virus vaccine (A/H1N1) adjuvanted with cholera toxin (left panel), non-adjuvanted HPV16 virus-like particles (VLP) (central panel), and H. pylori extract (right panel) were associated with protection against infection by the corresponding pathogen. Adapted from Song et al. (2008), Cuburu et al. (2009), and Rhagavan et al (2010)

genital challenge with HPV (Cuburu et al. 2009) (Fig. 5). Other recent experiments have shown that sublingual administration of an experimental *Helicobacter pylori* vaccine can effectively induce B and T cell responses in the stomach mucosa and protect mice against infection with *H. pylori* with an efficacy exceeding that achieved by orogastric immunization (Raghavan et al. 2010) (Fig. 5). Finally, sublingual immunization with experimental ETEC and *V. cholerae* whole cell vaccines, as well as purified fimbrial antigens, has proved efficient in giving rise to strong IgA antibody responses in the intestine, suggesting that this route may even be used as an alternative to the oral route for vaccination against enteric infections (Holmgren, unpublished data).

The exceptional ability of the murine sublingual mucosa to disseminate effector B and T cell responses to various mucosal tissues appears to be contributed by specialized dendritic cells residing in the sublingual epithelial and draining submaxillary (cervical) lymph nodes. These CCR7+ dendritic cells appear to respond to the chemokines CCL19 and CCL21 (Song et al. 2009) produced in the local microenvironment and to imprint B and T cells (including CTL precursors) to migrate to CCL28 that is expressed by epithelial cells in a variety of tissues, including the salivary glands, mammary glands, small and large intestines, respiratory tract and genital tract (Kunkel et al. 2003; Pan et al. 2000; Wang et al. 2000).

Controlled clinical trials are now being conducted to determine the safety and efficiency of this novel route of administration. The development of mucoadhesive formulations with enhanced permeabilizing properties to facilitate and prolong contact of vaccine antigens with the sublingual epithelium is likely to become a major milestone for the future emergence of sublingual vaccines.

4.3 Transcutaneous Vaccination

Another interesting route of vaccine administration relates to the use of skin-adhesive patches containing antigen and adjuvant (Glenn et al. 1998). This approach, called "transcutaneous immunization" has been shown to induce both systemic, intestinal and respiratory antibody responses in mice. The results of a clinical trial involving administration of *E. coli* heat-labile enterotoxin (LT) appear to be promising, and suggest that transcutaneous immunization with potent adjuvant-active antigens, such as LT, may also evoke both intestinal and systemic antibody responses in humans (Glenn et al. 2000). The ability of transcutaneous immunization to elicit intestinal antibody responses is intriguing and may relate to the observation that transcutaneous antigens co-administered with CT-like adjuvants induce IgA Ab-secreting cells that express CCR9 and CCR10 and can migrate to the small intestine (Chang et al. 2008).

4.4 Parenteral Vaccination

Finally, as discussed above (Fig. 1), parenteral vaccination routes may be effective for immunization against mucosal infections caused by pathogens which are taken up or penetrate the epithelium. For instance, parenteral vaccine-induced serum IgG antibodies can protect against intestinal pathogens either by preventing subepithelial microbial spread (e.g. shigellosis) or invasion through draining vessels (e.g. typhoid). In addition, parenteral administration might be used in tandem with mucosal vaccines, whether the latter are given by oral, nasal or sublingual route. Parenteral polio or cholera vaccines given as a booster have been found to stimulate antigen-specific S-IgA responses in naturally primed individuals although they did not induce any such response when given to immunologically naïve individuals. Thus, injectable and mucosal vaccines might synergize with each other in their protective profiles if given in tandem.

5 "Tropical Barriers" to Mucosal Vaccines

The oral polio vaccine (OPV), which was licensed more than 50 years ago, is a classic mucosal vaccine. In addition to its enormous impact in eradicating polio in most countries, this vaccine has served as a useful tool in elucidating the fundamental aspects of mucosal immunity in humans (Ogra and Karzon 1969; Ogra and Ogra 1973). Similar to the injectable, inactivated polio vaccine (IPV) but five times cheaper, OPV produces antibodies in the blood that prevent dissemination of poliovirus to the nervous system. However, unlike IPV, OPV also produces a local S-IgA immune response in the intestinal mucosa—the primary site of poliovirus entry. This intestinal immune response is the most critical property of the OPV,

since it can rapidly stop person-to-person transmission of wild poliovirus ("herd protection"), making mass campaigns with OPV a most powerful strategy for the global eradication of polio. This would not be feasible with the injectable IPV. However, at the same time, concerns have been raised after reports of low or no response to OPV in children from certain developing countries, even after giving as many as 10 OPV doses. Like the OPV situation, several live oral vaccines have performed poorly in developing countries compared with industrialized countries. This is attributed mainly to chronic environmental enteropathy (CEE), also called tropical enteropathy, which is characterized by disturbances in digestive and absorptive functions. Factors that may contribute to CEE include poor sanitation, overgrowth of intestinal flora, and histological changes characterized by inflammation and blunting of small intestinal villi leading to malabsorption. Children living under extreme poverty are especially sensitive.

Other factors that might hurt the performance of oral vaccines in developing countries include: deficiencies in nutrients such as vitamin A (retinol) and zinc, which can influence the response to oral adjuvants and vaccines by affecting discrete subpopulations of intestinal dendritic cells and T cells; persistent activation of the gut innate immune system by infectious agents, such as helminths and concomitant viral and bacterial infections; and interferences by maternal breast milk (breast milk from mothers of low socioeconomic status in developing countries contains high titers of antibodies to enteric pathogens that can interfere with oral vaccine "take").

Strategies for coping with the different causes of immune hyporesponsiveness to oral vaccines in developing countries include the co-administration of vaccines with agents that improve gut integrity, such as zinc, vitamin A, and possibly probiotics; withdrawal of breast milk shortly before oral vaccination; and treatment of helminths prior to oral immunization. It would be interesting to determine if vaccines administered by a non-intestinal route could overcome these gut barriers.

6 Surrogates of Mucosal Vaccine-Induced Immunity

As of today, there is no method that has been qualified by regulatory bodies for analysis of mucosal immune responses to vaccines. Traditional approaches, such as measurement of secretory antibodies in external secretions or in organ lavages using immunoassays have not gained general acceptance, having either met with problems of reproducibility (even for a given individual tested on several occasions in a single day) or their impracticality on a large scale (e.g. gut and bronchoalveolar lavages), especially in young infants and children.

Probably, the most challenging problem that these methods will continue to face is the inherent compartmentalization of immune responses induced by mucosal immunization. Thus, immune responses measured in one mucosal tissue do not faithfully reflect responses induced in another.

Several approaches are now being developed based on the improved knowledge of mechanisms governing dissemination of mucosal immune responses and especially of mucosal plasmablasts. One such approach utilizes the known ability of recently activated antibody-secreting plasmablasts to circulate in blood after antigen/vaccine exposure, regardless of where these cells were activated. Combined immunomagnetic cell-sorting and ELISPOT assay can now be performed on small samples of whole blood (without gradient enrichment for mononuclear cells) and allows for partitioned measurement of systemic and mucosal antibody responses to vaccines by detecting antigen-specific plasmablasts with a specific mucosal pedigree (e.g. $\alpha 4\beta 7$, CCR10). This approach may in the future be expanded to cells with defined mucosal tissue tropism, such as cells expressing markers specific for the small intestine, large intestine, lung, or genital tract.

7 Perspectives

Better knowledge of human mucosal immune responsiveness during early life is required to establish the usefulness of different routes of vaccine administration against pathogens encountered by neonates and young infants from developing and industrialized countries. To explore the impact of environmental factors (tropical enteropathy, malnutrition and maternal factors) on mucosal responses to vaccines administered by different routes in developing countries, studies should be conducted with licensed killed and live mucosal vaccines, and for comparison, also with live and killed parenteral vaccines. Animal models could also be helpful in exploring the influence of these factors on mucosal immune responsiveness to antigens and adjuvants administered by these different routes.

The choice of mucosal vaccination route will impact overall vaccine design, including the selection of appropriate adjuvants and formulations, and thus, manufacturing issues. When compared with most licensed injectable vaccines, it is interesting to note that currently, there are no pure subunit vaccines formulated and licensed for mucosal administration. Because most injectable subunit vaccines are given with an adjuvant, a further challenge in the field will be the development of adjuvants to enhance the potency of future subunit vaccines administered by different mucosal routes.

Dearly needed are standardized, validated assays that do not require large sample volumes (especially in young children), and reference reagents for large scale measurements of mucosal immune responses to vaccines. Further research is needed to understand the mechanisms contributing to the generation and maintenance of mucosal adaptive B cell and T cell memory responses.

However, these challenges could be met with expanded use of animal models, multi-disciplinary efforts between basic scientists and clinical vaccinologists, and, most importantly, by implementing experimental systems using both animal models and human clinical trials to address the gaps in our understanding of the human mucosal immune system.

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