Chapter 2
Mucosal Immunology and Oral Vaccination

Ana Lilia García-Hernández, Néstor Rubio-Infante and Leticia Moreno-Fierros

Introduction

The mucosal surfaces of the gastrointestinal and respiratory tracts represent the principal portals of entry for most infectious agents. Hence, the development of vaccination strategies capable of inducing protective immune responses at the mucosal sites is a priority. Since the mucosal surfaces are exposed to a wide variety of antigens, the mucosal immune system has to discriminate between harmful and harmless inoffensive or beneficial antigens. For this reason, the mucosal immune surfaces are highly regulated by a complex interplay of regulatory mechanisms capable of eliciting strong immune responses against pathogens and protecting the body as well as preventing the induction of strong immune responses against dietary proteins, commensal bacteria, or environmental inoffensive antigens, which can lead to chronic diseases (Mowat 2003; Pabst and Mowat 2012).

Mucosal surfaces are protected from external attacks by physicochemical defense mechanisms comprising innate and adaptive mucosal immune systems. Epithelial barriers on the 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. The intestinal mucosa is covered by only a single cell layer (type 1 epithelium), whereas multilayered squamous epithelia line the oral cavity, pharynx, esophagus, and urethra (type 2 epithelium); and the airway and vaginal linings vary from pseudo-stratified to simple epithelium (Box 2.1; Pavot et al. 2012).

A major goal in vaccine design comprises the induction of protective lasting immune responses against potential pathogens on the mucosal surfaces. These responses are most effectively induced by the administration of vaccines onto mucosal surfaces through oral, nasal, rectal, or vaginal routes, when compared with those induced by parenteral routes (Neutra and Kozlowski 2006). In addition, mucosal vaccines offer

L. Moreno-Fierros (✉) · A. L. García-Hernández · N. Rubio-Infante
Inmunidad en Mucosas, UBIMED, FES-Iztacala, Universidad Nacional Autónoma de México, Avenida de los Barrios 1, Tlalnepantla, 54090, Mexico
e-mail: lemofi@servidor.unam.mx

S. Rosales-Mendoza (ed.), *Genetically Engineered Plants as a Source of Vaccines Against Wide Spread Diseases,* DOI 10.1007/978-1-4939-0850-9_2,
© Springer Science+Business Media New York 2014

Box 2.1 Mucosal Immunity Is Mediated by Different Lines of Defense

(1) IgA, antimicrobial peptides (such as defensins, angiogenins, defensin-like peptides, and catelicidins released by enterocytes, Paneth cells, as well as by intraepithelial lymphocytes), and mucus glycoproteins
These components are the first line of defense forming a mucosal layer and dismiss the penetration of most bacteria. IgA neutralizes pathogens while antimicrobial peptides can reach sufficient levels to mediate bacterial lysis in crypts (Mowat 2003).
(2) Epithelial barrier
The second barrier of defense comprising the monolayer of the epithelial cells (ECs) and the upregulated permeability provided by tight junctions through these cells, which are formed by a single epithelial stem cell; absorptive enterocytes, microbicidal factor-producing Paneth cells, mucus-producing goblet cells, and hormone-producing enteroendocrine cells protect against invasion of luminal microbes into the sterile tissues (Brandtzaeg et al. 1999).
(3) Lamina propria
It is considered the final barrier before systemic immunity and contains distinct lymphoid structures that can detect and restrain microbes through the action of dendritic cells, macrophages, lymphoid cells, stromal cells, and plasmatic cells (Coombes and Powrie 2008).

needle-free delivery, thereby improving accessibility, safety, and cost-effectiveness. Mucosal vaccines are also advantageous when compared with systemic vaccines from a production and regulatory perspective. For example, vaccines for oral use do not require extensive purification from bacterial by-products since the gut is already heavily populated by bacteria, whereas the same vaccine formulation injected parenterally would have unacceptable endotoxin levels (Lycke 2012). Nevertheless, the vast majority of vaccines in use today are administered by intramuscular or subcutaneous injections, where a proper control on dosage can be accomplished. By contrast, the dose of a mucosal vaccine that enters the body is not accurately determined. Moreover, several challenges to achieve successful mucosal vaccination still prevail, comprising poor induction of mucosal immunity, limited understanding of protective mechanisms and cross talk between mucosal compartments, and the availability of safe and effective mucosal adjuvants as well as delivery systems. Our understanding of mucosal immunity and development of mucosal vaccines has lagged behind, in part because the induction and measurement of mucosal immune responses are more complicated than those elicited by parenteral routes. As a result, only a few mucosal vaccines have been approved for human use worldwide. Among these, oral vaccines against poliovirus, *Salmonella typhi, Vibrio cholerae,* and rotavirus, and a nasal vaccine against influenza virus can be mentioned (Pavot et al. 2012; Woodrow et al. 2012). However, research and testing of mucosal vaccines are currently accelerating, stimulated by new information on the mucosal immune system and by the threat of the mucosally transmitted virus, such as the Human

immunodeficiency virus (HIV). Fortunately, current research is providing new insights into the function of mucosal tissues and the interplay of innate and adaptive immune responses that result in immune protection at mucosal surfaces (Neutra and Kozlowski 2006).

To better understand the limitations and challenges for developing successful oral vaccines, some general anatomical and functional characteristics of the mucosal immune system will be described in this chapter, particularly of the one associated with the intestinal mucosa. Current strategies for successful mucosal vaccination will be further analyzed, highlighting the advantages of oral vaccines.

Organization of the Mucosal Immune System

The mucosal immune system can be divided into inductive and effector sites. The first ones are constituted by organized mucosa-associated lymphoid tissue (MALT) as well as mucosa-draining lymph nodes. The latter are represented by the lamina propria (LP), the stroma of exocrine glands, and surface epithelia.

MALT comprises multiple compartments including the gut-associated lymphoid tissue (GALT), which is the largest human mucosa and immunologic organ in the body. The gastrointestinal mucosa is associated to specialized components of the innate and adaptive immunity (specific antigen recognition, effector and memory functions) that protect the host against pathogens, control responses to food components, and mediate tolerance against harmful antigens (Holmgren and Czerkinsky 2005).

In the GALT, the organized tissues responsible for the induction phase of the immune response comprise the Peyer's patches (PP) and mesenteric lymph nodes (MLNs), as well as smaller, isolated lymphoid follicles (ILFs), which have the appearance of microscopic PP and are distributed throughout the walls of the small and the large intestines. The diffuse lymphoid tissue of the effector sites at the intestinal mucosa consists of lymphocytes scattered throughout the epithelium and LP of the mucosa (Fig. 2.1).

Characteristics of the Organized Inductive Lymphoid Tissues

Organized lymphoid tissues such as the PP consist of collections of large B cell follicles and intervening T cell areas. The lymphoid areas are separated from the intestinal lumen by a single layer of columnar epithelial cells, known as the follicle-associated epithelium (FAE), and a more diffuse area immediately below the epithelium, known as the subepithelial dome (SED; Fig. 2.1). The FAE differs from the epithelium that covers the villus mucosa as it has lower levels of digestive enzymes and a less pronounced brush border, and it is also infiltrated by large numbers of B

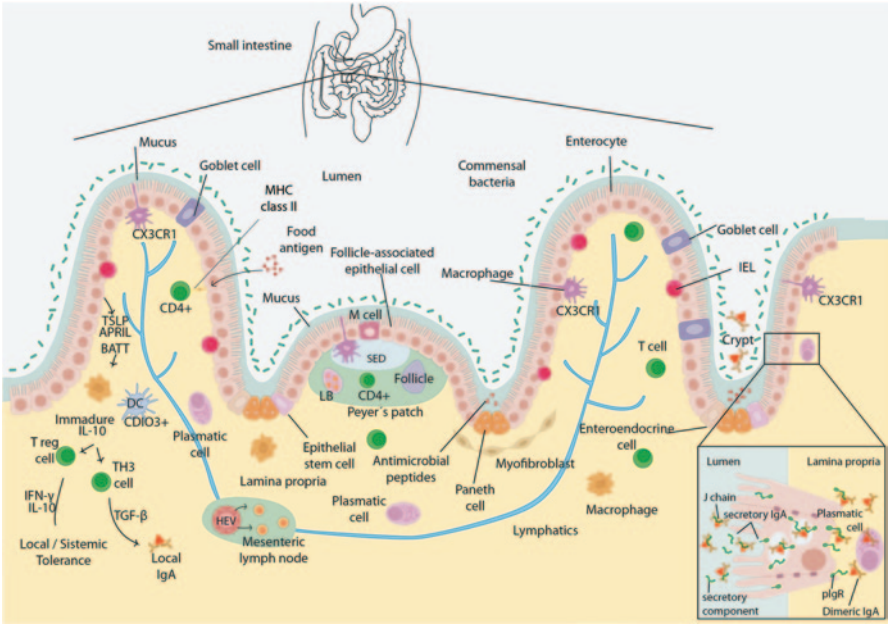

Fig. 2.1 Anatomy and homeostasis of the intestinal immune system. The gut-associated lymphoid tissue (GALT) can be divided into inductive and effector sites, which consist of organized and diffuse lymphoid tissues, respectively. The organized tissues are the Peyer's patches (PP) and mesenteric lymph nodes (MLNs), as well as smaller, isolated lymphoid follicles. The effector tissues consist of lymphocytes scattered throughout the epithelium and lamina propria (LP) of the mucosa. A single layer of intestinal epithelial cells (IECs) provides a physical barrier that separates the commensal bacterial in the intestinal lumen from the underlying LP. The IECs lining the lumen are bathed in nutrients, commensal bacteria, IgA, and goblet cell-produced mucus. These IECs differentiate into villous or colonic enterocytes, which absorb nutrients (small intestine) and water (colon). Progenitor IECs differentiate into both enteroendocrine cells, which secrete enteric hormones, and Paneth cells at the base of the small intestinal crypts. Paneth cell granules contain high concentrations of α-defensins. Certain subsets of T cells (intraepithelial lymphocytes, IEL) and macrophages cells CX3CR1+ localize between the IECs. In the small intestine, about 80% of IEL are CD8+ lymphocytes and about 70% of CD4+ lymphocytes is present in the LP. The specialized epithelium termed follicle-associated epithelium contains microfold (M) cells that overlie the subepithelial dome (SED) of the organized lymphoid tissue PP consist of a rich zone of B lymphocytes in an area termed follicles, and around them is a thymus-dependent area (TDA), which is rich in CD4+ T lymphocytes. The LP, contains B cells (especially sIgA-producing plasmatic cells), T cells CD4+, stromal cells, and antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) CD103+. Oral tolerance is essential to maintain homeostasis. Food proteins and products of commensal bacteria are taken up by IECs which express MHC II, but do not express the co-stimulatory molecules; thus, they contribute to oral tolerance induction. IECs also produce chemokines like APRIL and B-cell-activating factor (BAFF), which promote B cell recruitment in the LP and class switching in response to TLR signaling, and thymic stromal lymphopoietin (TSLP), the transforming growth factor-β (TGF-β), retinoic acid (RA), and possibly other factors that promote the induction of regulatory T (Tregs) cells. Specific subsets of intestinal DCs CD103+ express RA-synthesizing enzymes, and in the presence of TGF-β, induce the differentiation of naïve T_R cells, Foxp3+. RA also programs DCs to imprint gut-homing properties. These committed T_R cells home back to the intestinal LP through high endothelial venules (HEVs), where they undergo secondary expansion under the influence of interleukin-10 (IL-10) produced by CX3CR1+ macrophages. These T cells differentiate into Treg cells, and also produce IL-10 and interferon-γ (IFN-γ) and/or T helper (T_H) 3 cells, which produce TGF-β-favoring oral tolerance

cells, T cells, macrophages, and dendritic cells (DCs). The most notable feature of the FAE is the presence of microfold (M) cells, which are specialized enterocytes that lack surface microvilli and the normal thick layer of mucus. Antigens are taken up by absorptive epithelial cells or specialized epithelial M cells in mucosal inductive sites, or alternatively, can be directly captured by "professional" antigen-presenting cells (APCs), which include DCs, B lymphocytes, and macrophages. Antigen-charged DCs further process and present antigens to T cells located at the interfollicular areas within the PP. Primed lymphocytes exit through the draining lymphatics to the MLNs, where they reside for an undefined period of further differentiation before they migrate into the bloodstream through the thoracic duct and finally accumulate in the mucosa (Holmgren and Czerkinsky 2005; Mowat 2003).

Priming of T and B cells in these inductive tissues and selective homing to mucosal sites lead to either efficient local immune responses or tolerance. However, how the intestinal captured antigens can also induce systemic priming or tolerance involves complex mechanisms. The MLNs are considered alternative sites where T cell priming might occur and explain the induction of local and systemic immunity or tolerance by the oral route. The antigens might reach the MLNs via the draining lymph (Fig. 2.2) or as a result of APCs located in the LP that have taken up antigens either directly from the lumen or from APCs that have acquired unprocessed antigens from M cells, and then migrated to MLNs. T cells that are primed in the MLNs are further differentiated, and then migrate to the mucosa to mediate local immune responses. In addition, since the MLNs can act as a crossover point between the peripheral and systemic immune systems, this pathway might also explain the induction of systemic immunity or tolerance in response to intestinal antigens (Mowat 2003).

Mucosal Effector Tissues

The diffuse lymphoid tissues are mainly associated with effector responses that are initiated from the organized lymphoid tissues. These diffuse lymphoid tissues are mainly composed of lymphocytes residing as intraepithelial lymphocytes (IELs) in the mucosal epithelium in addition to numerous lymphocytes present in the LP, which is the connective tissue directly underlying the mucosal epithelium.

Intraepithelial Lymphocytes

The IELs that reside within the epithelium of the intestine form one of the main branches of the immune system by their direct contact with the enterocytes and by their immediate proximity to antigens in the gut lumen. As IELs are located at this critical interface between the core of the body and the outside environment, they must balance protective immunity with an ability to safeguard the integrity of the epithelial barrier, as failure of this function would compromise homeostasis (Cheroutre et al. 2011).

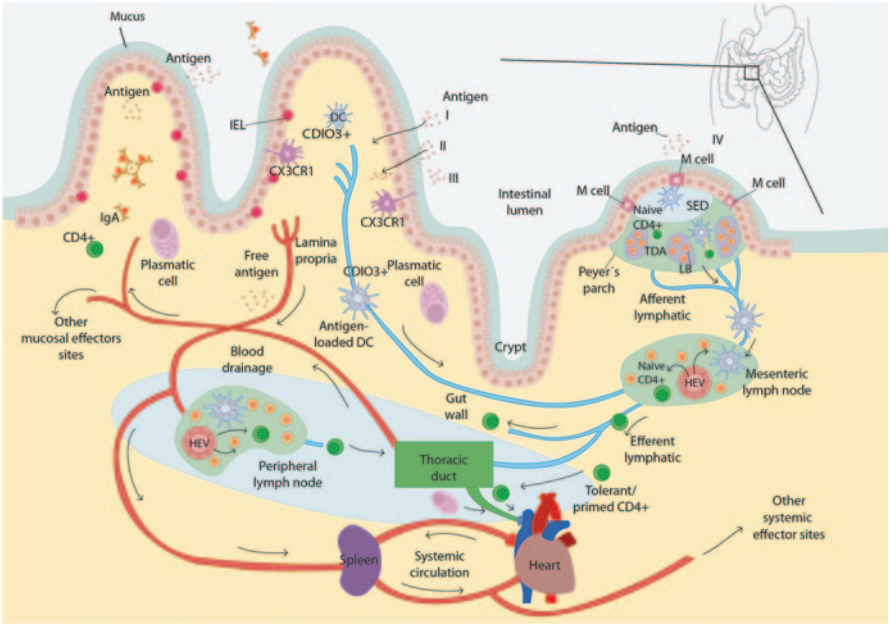

Fig. 2.2 Antigen uptake in gut-associated lymphoid tissue (GALT). The antigen might enter GALT through different parts of the intestine. Epithelial cells can acquire soluble antigens that have diffused through epithelial tight junctions (*I*) or have been transferred across epithelial cells by transcellular routes (*II*). CX3CR1+ macrophages can also capture luminal antigens by extending processes through the epithelial layer, and they may pass this to neighboring CD103+ dendritic cells (DCs) (*III*). Also, the antigen might enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (*IV*) and after transfer to local CD103+ DCs; the antigen might also gain direct access to the bloodstream from the gut and interact with T cells in peripheral lymphoid tissues. The antigen taken up into Peyer's patches (PP) or lamina propria may enter the bloodstream via the portal vein, first reaching the liver before it becomes distributed into the circulation. Free antigen taken up into afferent intestinal lymph will pass through the mesenteric lymph nodes and eventually enter the bloodstream via the thoracic duct. Once the antigen is sampled by M cells, it is delivered across the epithelial barrier directly to subepithelial DCs that subsequently process and present antigen locally to T cells located at the interfollicular areas within the PP. Alternatively, antigen or antigen-loaded DCs from the PP might gain access to the draining lymph, with subsequent T, B cell recognition in the mesenteric lymph nodes (MLNs). In all cases, the antigen-responsive CD4+ T cells or plasmatic cells acquire expression of the α4β7 integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph, and, after entering the blood stream through the thoracic duct, exit into the mucosa through the vessels in the LP. T cells and plasmatic cells, which have recognized antigen first in the MLN, may also disseminate from the bloodstream throughout the peripheral immune system. Plasmatic cells produce local sIgA and systemic IgG. Since T cells and plasmatic cells migrate through the circulation, integrin and chemokine signals direct their emigration into tissues. In this manner, imprinted T cells and plasmatic cells have a specific key that allows access to restricted tissues

IELs essentially comprise antigen-experienced T cells belonging to both T cell receptor-γδ (TCRγδ)$^+$ and TCRαβ$^+$ lineages, but are extremely heterogeneous, and the various IEL subsets are distributed differently in the epithelia of the small and large intestines probably influenced by the distinct digestive functions and the physiological conditions between both intestines. In the small intestine, IELs are almost exclusively T cells and include a significant proportion of TCRγδ$^+$ cells (60%). IELs constitutively express CD103 (also known as the αE integrin), which interacts with E-cadherin on intestinal epithelial cells, and most of them, especially in the small intestine, express CD8αα homodimers, which is a hallmark of their activated phenotype. The majority of IELs express activation markers, such as CD44 and CD69; contain abundant cytoplasmic granules responsible for cytotoxic activity; and can express effector cytokines, such as interferon-γ (IFNγ), interleukin-2 (IL-2), IL-4, or IL-17. Furthermore, IELs characteristically express both activating and inhibitory types of innate natural killer (NK) cell receptors, which typify them as stress-sensing (activated) yet highly regulated (resting) immune cells (Cheroutre et al. 2011). IELs play an important role in controlling the entrance of commensal bacteria after epithelial damage via the release of antimicrobial peptides and promoting the repair of injured gut epithelia. IELs express a limited diversity of antigen receptors, keep in a heightened state of activation, and thus avoid the need for a priming step before full activation.

Lamina Propria Lymphocytes

Lymphocytes in the LP include mainly the CD4+ T cells and also an important population of plasma cells, which are B lymphocytes that are mainly IgA in type I mucosal tissues like the one present in intestines. An important characteristic of the mucosal adaptive immune response is the local production and secretion of dimeric secretory immunoglobulin A (sIgA), which, unlike other antibody isotypes, are resistant to degradation in the protease-rich external environments of mucosal surfaces. sIgA is secreted as a dimer across the mucosal epithelium by an active transport mechanism using the polymeric Ig receptor (pIgR). sIgA has multiple roles in mucosal defense as it can bind and neutralize pathogens or toxins in the gut despite the presence of active digestive enzymes. It promotes the entrapment of antigens or microorganisms in the mucus preventing direct contact of pathogens with the mucosal surface, a mechanism that is known as "immune exclusion." Protection of mucosal surfaces by sIgA can also be mediated by intracellular neutralization of pathogens that have invaded the epithelial cells when the sIgA is transported by the pIgR. In addition, antigens can be excreted through the secretion of sIgA joined to the antigens, which is released into the mucosal lumen (Strugnell and Wijburg 2010). Moreover, sIgA-mediated blockade is also a key element in the intestinal homeostasis as it reduces inflammatory activity of the microbiota (Mantis et al. 2011).

Although the adaptive humoral immune defense at mucosal surfaces is mainly mediated by sIgA, locally produced IgM and IgG in the respiratory tract and in the

genitourinary mucosa and serum-derived IgG can also contribute significantly to the mucosal immune defense (Neutra and Kozlowski 2006; Iwasaki 2010).

The lymphocytes that enter the mucosa redistribute into distinct compartments. The functions of mucosal T cells are still largely undefined, but cells with a "memory" or "effector memory" phenotype predominate in both the epithelium and the LP, indicating that these have been exposed to an antigen. In the LP of the intestine, CD4+ T cells are of particular importance in regulating local immune responses. LP CD4+ T cells might be regulatory T (Tregs) cells and therefore responsible for maintaining local tolerance to environmental antigens. These produce large amounts of cytokines, particularly IFN-γ, but also IL-4 and IL-10.

LP CD8+ T cells can also have potent cytotoxic T lymphocyte (CTL) activity. Some of these antigen-experienced LP T cells might be true effector cells, and might help local B cells to produce IgA "effector memory" cells, as indicated by the findings supporting that antigen-specific memory CD4+ and CD8+ T cells accumulate preferentially in non-lymphoid tissues, particularly at the intestinal mucosa (Shale et al. 2013; Mowat 2003).

Intestinal CD4+ T cells are essential mediators of immune homeostasis and inflammation. Multiple subsets of CD4+ T cells have been described in the intestine, which represents an important site for the generation and regulation of cells involved in immune responses both within and outside of the gastrointestinal tract. Among intestinal lymphocytes, CD4+ T cells represent a major population implicated in mediating diverse host-protective and homeostatic responses (Shale et al. 2013).

T cell populations can be broadly functionally divided into effector and regulatory populations. The lack of inflammation in the majority of individuals, despite the enormous microbial and antigenic load within the intestine, clearly demonstrates the dominance of regulatory mechanisms in the steady state, condition in which IL-17 cells are the dominant Th17A single positive CD4+ T cells, and preferentially locate the LP of the small intestine and, to a lesser extent, the colon and intestine of adult mice. Interestingly, expansion of Th17 cell populations in the small intestine may occur in the setting of extraintestinal infections or autoimmune diseases without detectable mucosal inflammation. In the steady state, the presence of dominant, suppressive, and regulatory mechanisms restrains innate and adaptative responses. Functionally specialized subsets of CD4+ T cells play an important role in the regulation of intestinal immune responses. The concept of an important functional role for CD4+ Treg cells in maintaining intestinal homeostasis was established originally in mice, where the ability of CD4+ CD25+ Treg cells to prevent disease in the T cell transfer model of colitis was described. A number of subsets of T cells possessing regulatory or suppressive activity have now been characterized, but those expressing the transcription factor Foxp3 and IL-10-producing cells appear to be of particular functional importance in intestinal homeostasis and in the control of inflammation. In comparison with systemic immune compartments, the intestine is enriched with the presence of Treg cells. Although IL-10+ Foxp3+ Treg cells are also found in abundance in the small intestinal LP, a sizable fraction of IL-10+ CD4+ T cells in this location do not express Foxp3, exhibiting a Tr1 phenotype. TGF-β plays a critical role in the development and function of Treg cells, including

Foxp3 + and Tr1 cells. Cells co-expressing RORγτ and Foxp3 are found in intestinal tissues. Notably, both Treg cells and Th17 cells require TGF-β for their development, and the presence or absence of further factors, including the STAT3-activating cytokines, IL-6 and IL-23, may determine the balance of these populations in the steady state or inflamed intestine. Interestingly, intestinal CD4 + T cell subsets are also regulated by environmental factors. The microbiota directs the accumulation of both Treg cells and Th17 cells in the intestinal LP (Shale et al. 2013).

Intestinal APCs

Together with the epithelial barrier, APCs and IELs are located at the first line of defense. After sensing pathogens, these cellular types release cytokines, antimicrobial peptides, and chemokines as defense or activate and recruit immune cells that furthermore can phagocytose and kill pathogens.

Antigen sampling strategies are adapted to the diverse epithelial barriers that cover mucosal surfaces throughout the body, but all involve collaboration with APCs. Myeloid APCs of the intestine are a heterogeneous population consisting of DCs and macrophages (Pabst and Mowat 2012; Geissman et al. 2010; Scott et al. 2011; Manicassamy and Pulendran 2009). These populations are strategically positioned with the LP and in organized lymphoid structures, and exhibit a number of adaptations associated with their dual role in tolerance and immunity in the intestine. Myeloid APCs might congregate immediately under epithelia, migrate into the epithelial layer, and even extend dendrites into the lumen to capture antigens. DCs can act as a bridge with the adaptive immune system through their ability to acquire antigen in the intestine and migrate to the MLN where they prime the activation of cognate naive T cells. In addition to presenting antigens, intestine-derived DCs are specialized in their ability to prime T cell responses that are focused on the intestine through the upregulation of gut-homing molecules on the responding T cell surface (Box 2.2).
At sites of organized mucosal lymphoid tissues, specialized M cells in the lymphoid FAE sample and deliver antigens across the epithelial barrier directly to subepithelial

Box 2.2 Importance of Mucosal Homing in the Choice of Mucosal Vaccination Route

After the initial exposure to antigen, lymphocytes leave PP or other mucosal inductive sites and migrate into mucosal tissues, including the intestines, lungs, nasal passages, and urogenital tract. These lymphocytes home to the LP or mucosal epithelium where they exert effector activities such as antibody synthesis or killing virally infected cells. The preferential migration of mucosal stimulated lymphocytes to other mucosal sites throughout the body gave rise to the idea of a “common mucosal immune system.” However, it is now apparent that the mucosal immune system is highly compartmentalized

and thus lymphoid responses preferentially migrate into tissues where the response was induced. Therefore, the compartmentalization within the mucosal immune system places constraints on the choice of vaccination route for inducing effective immune responses at the desired sites (Holmgren and Czerkinsky 2005). Therefore, in order to induce and regulate protective immune responses at the appropriate mucosal sites, depending on the invading route of a particular pathogen, it is required to understand the biological basis of the mucosa compartmentalization (Brandtzaeg et al. 1999).

The capacity for selective migration of effector and memory T and B cells back to the original challenge site—the concept of tissue-specific homing—or to the distinct mucosal sites, depends on the differential expression of adhesion molecules on the lymphocyte cell surface as well as on the vascular endothelium. Whereas naive T cells express adhesion molecules and chemokine receptors that restrict their migration mainly (but not entirely) to organized lymphoid tissue, activated memory T cells downregulate these lymphoid-tissue-homing receptors and upregulate tissue-specific adhesion molecules and chemokine receptors that target their migration to non-lymphoid tissues (Kunkel and Butcher 2002).

This imprinting of tissue-homing properties is best described for the gut and skin. Priming of T and B cells in PP and mesenteric lymph nodes preferentially induces the expression of α4β7 integrin and CC-chemokine receptor 9 (CCR9), whereas T cells that are primed in peripheral lymph nodes upregulate cutaneous leukocyte antigens, CCR4 and CCR10. Endothelial cells of postcapillary venules in the intestinal mucosa constitutively express ligands for α4β7-integrin and CCR9, namely mucosal addressin cell-adhesion molecule 1 (MADCAM1) and CC-chemokine ligand 25 (CCL25), also known as thymus-expressed chemokine (TECK), which is expressed selectively by small bowel epithelial cells, allowing lymphoid cells that are induced in intestinal lymphoid tissue to enter this mucosal effector site. Importantly, recent investigations suggest that antigen-presenting DCs process and "interpret" locally produced metabolites to program tissue-specific lymphocyte homing. In the case of GALT, resident DCs metabolize vitamin A to retinoic acid, which stimulates α4β7-integrin and CCR9 expression by T cells; and in the skin, local DCs use metabolites of vitamin D3 to program T cells in recurrent laryngeal nerves (RLNs) (Kunkel and Butcher 2002).

The identification of α4β7-integrin and CCR9 as mucosal homing receptors interacting with MADCAM1 and CCL25, respectively, was considered the molecular explanation for the fact that mucosal vaccination is required for protection against mucosal infections, whereas parenteral vaccines are generally ineffective to induce mucosal immunity. It must be taken into account that recruitment of lymphoid cells into target tissues requires specific chemokine recognition and adhesion-receptor engagement. The high degree of compartmentalization among the distinct mucosal sites relies on the use of distinct

set of mucosal homing receptors. Indeed, there are distinct tissue-trafficking patterns for both B and T cells that depend on their site of induction. For example, plasma-cell precursors that are primed in respiratory tract lymphoid tissues home to the tracheal and bronchial mucosa, express only low levels of the gut-homing molecules, α4β7-integrin and CCR9, but express high levels of α4β1-integrin and CCR10. Importantly, the counterparts of α4β1-integrin and CCR10, vascular cell adhesion molecule 1 (VCAM1) and CC-chemokine ligand 28 (CCL28), respectively, are constitutively expressed by airway mucosal endothelial cells. Lung T cells also express distinct phenotypes and lack intestinal-homing molecules. Moreover, nasal and vaginal cells also express a phenotype that is distinct from gut-homing T cells (Iwasaki 2010; Holt et al. 2008).

DCs that subsequently present antigen locally in adjacent mucosal T cell areas. Gut APCs can be found scattered in the LP of the gastrointestinal tract (small and large intestine) and in the radial muscle layer. The intestinal DCs play the most important role as professional APCs, since they express up to 100 times more major histocompatibility complex (MHC) molecules and are more effective in priming naïve T cells in the organized lymphoid tissue (PP and ILFs), MLN, and LP. DCs are considered the primary inductors to T-cell-dependent IgA responses (Manicassamy and Pulendran 2009). In mice, APCs can be grouped based on the expression of CD103 (α-E integrin) and CX3CR1 (the receptor of fractalkine). CX3CR1+ cells derive from a monocytic precursor that may be recruited in response to the microbiota and have been described to drive the development of Th17 cells in vitro, presumably via a flagellin or ATP-dependent pathways. In particular, CX3CR1+ CD11b+ CD11c+ DCs and CD103+ DCs have been well characterized, while in homeostatic conditions CD103+ DCs derive from circulating DC precursors (pre-DCs) and have tolerogenic potential. These cells can also imprint T cells with gut-homing properties in both mice and humans (Scott et al. 2011). Regarding CX3CR1+ APCs, it is not yet clear what their function is, as they are incapable of migrating out of the gut and low-effective APCs (Pabst and Mowat 2012). However, a recent report has shown that these cells acquire migratory properties in the absence of the microbiota.

Routes of Antigen Uptake and Induction of Mucosal Immune Responses

The immunological consequences of oral administration of antigen ultimately depend on where and how antigen is taken up and presented to T cells. Most soluble, non-adherent antigens are taken up at low levels, and generally induce immune tolerance (Pabst and Mowat 2012).

The possible routes of antigen uptake are outlined in Fig. 2.2. The conventional pathways by which it is assumed that this might occur comprise the uptake of particulate antigen into PP or isolated lymphoid tissues through M cells, and also by

the alternative routes of antigen uptake which might be of relevance. These are the following: the transfer of intestinal antigen and/or APCs from the PP or mucosal LP through the draining lymph to the MLNs, followed by local presentation to naïve T cells; blood-borne dissemination of antigen to peripheral lymphoid tissues; transfer of antigen to the liver through the portal vein; and local presentation of antigen to T cells by enterocytes or professional APCs in the LP (Pabst and Mowat 2012).

Villus enterocytes participate in a route for antigen uptake and also have been proposed as intestinal APCs directed to CD4+ T cells. This function was proposed since enterocytes are MHC class-II positive in most species, but normally do not express the co-stimulatory molecules that are required for full T cell activation; thus, they were considered as good candidates for tolerogenic APCs in vivo. However, it is improbable that presentation of antigen by enterocytes to adjacent CD4+ T cells might help to explain local tolerance, since naive CD4+ T cells are located in the organized lymphoid tissues and are rarely located in the LP. In addition, LP T cells do not migrate out of the gut, and, therefore, it seems unlikely that this pathway could contribute to systemic tolerance (Mowat 2003).

Role of Epithelial Cells in Mucosal Defense

In the gastrointestinal tract, a single layer of epithelial cells joined by tight junctions faces a complex luminal environment rich in microorganisms. Epithelia and their associated glands (such as the salivary glands) produce non-specific or innate defenses, including mucins and antimicrobial proteins. Nevertheless, foreign antigens and microorganisms frequently breach the epithelial barrier, and mucosal tissues are sites of intense immunological activity. In the intestinal mucosa, dispersed lymphoid cells and APCs are particularly abundant. Epithelial cells are active participants in mucosal defense since they function as sensors that detect dangerous microbial components through pattern recognition receptors such as Toll-like receptors (TLRs), and they respond by sending cytokine and chemokine signals to the underlying mucosal cells, such as DCs and macrophages, to trigger innate, non-specific defenses and promote adaptive immune responses. In the intestine, where bacteria are abundant, epithelial cells, together with IELs and the underlying phagocytic cells, can modulate and dampen these signals to prevent undesirable responses to non-threatening nutrients and the normal intestinal flora that could lead to mucosal inflammation (Artis 2008; Rescigno 2011).

Regulation of the Intestinal Immune System and Oral Vaccination

The mucosal surfaces are continually exposed to a wide variety of foreign antigens. As many of them do not represent any risk to the body, such as food proteins and commensal bacteria, maintaining the homeostasis and preventing damage or mucosal disorders, such as allergy and mucosal inflammatory diseases, are accomplished by sophisticated regulatory mechanisms that had evolved at these sites. The gastrointestinal tract is the largest reservoir of immune cells in the body; thus, the intestinal immune system is also considered the most complex part of the immune system. The mucosal immune system is able to distinguish between pathogenic and commensal bacteria or other inoffensive antigens and mount the appropriate immune responses, either effective protective immunity or regulatory responses. For example, protecting the gastrointestinal tract from invading pathogens requires strong protective immunity. By contrast, active immunity against non-pathogenic materials would be wasteful, and hypersensitivity responses against dietary antigens or commensal bacteria can lead to chronic inflammatory disorders such as coeliac disease and inflammatory bowel disease, respectively. Therefore, the default responses to most soluble non-toxic antigens are either mucosal immune tolerance or non-inflammatory responses (Pabst and Mowat 2012).

Particularly, the usual response to harmless gut antigens consists of the induction of local and systemic immunological tolerance, known as oral tolerance (Mowat 2003). In addition to its physiological importance, this phenomenon can be exploited for the treatment of autoimmune and inflammatory diseases, but it is also an obstacle when the development of recombinant oral vaccines is pursued. For these reasons, understanding the processes that determine the immunological consequences of oral administration of antigens is of key importance.

Basis of Tolerance Induction at the Mucosal Tissues

It has been proposed that specific features of mucosal tissues favor the induction of tolerance in terms of production of IgA antibodies and, to a lesser extent, T helper 2 (Th2) cell responses. However, several features of mucosal tissues might contribute to these effects, including a unique ontogeny and anatomical patterning, specialized cells and organs that are involved in the uptake of antigen, distinctive subsets of APCs, and several unusual populations of B and T cells. In addition, the migration of lymphocytes to the intestine is controlled by a series of unique adhesion molecules and chemokine receptors (Pabst and Mowat 2012).

Challenges in Oral Vaccine Design and Current Strategies to Achieve Mucosal Immune Responses

Mucosal vaccines that are orally administered face the same gauntlet of host defenses as do microbial pathogens: They are diluted in mucosal secretions, captured in mucus gels, attacked by proteases and nucleases, and excluded by epithelial barriers, and thus relatively large doses of vaccine are required, and it is difficult to determine with accuracy the dose that crossed the mucosa (Neutra and Kozlowski 2006).

Several strategies have been developed to advance the development of mucosal vaccines, including the use of diverse antigen-delivery systems and mucosal adjuvants. The main characteristics of these strategies, including advantages and limitations, are summarized in Table 2.1 (see Box 2.3).

Box 2.3 Routes of Mucosal Vaccination

Nasal route

Intranasal administration is an attractive immunization route due to the following features: Nasal mucosa is a practical site that lacks acidity, the secreted enzymes are limited, and small mucosal surface area requires a low dose of antigen. Furthermore, the nose is highly vascularized, easily accessible, and can be used for global immunization of large populations. It is well established that vaccines administrated by nasal route can induce both mucosal and systemic immune responses, preferentially if the vaccine is based on attenuated live cells or an antigen is accompanied by an adjuvant. This has been confirmed in nasal immunization of humans against diphtheria, tetanus, influenza, and *Streptococcus mutans*. Furthermore, potent responses in the respiratory and genital tracts can be induced by intranasal immunization as a result of the induction sites in nasopharynx-associated lymphoid tissue (NALT) that contains all of the immunocompetent cells required for the induction of antigen-specific immune responses. Nasal vaccination has proven to be an effective regimen for the stimulation of the respiratory immune system and can elicit both humorla and cellular responses.

Different nasal vaccine systems in humans and animals have been described. In fact, an intranasal live influenza virus vaccine has been approved by the Food and Drug Administration (FDA). This vaccine is safe, well tolerated, and up to 93 % effective against culture-confirmed influenza (Rappuoli et al. 2011; Woodrow et al. 2012; Pavot et al. 2012; Cheroutre et al. 2011; Yuki and Kiyono 2009).

Vaginal route

Vaginal mucosa is characterized by a type II epithelium that does not have histologically demonstrable MALT, but these mucosal surfaces in the female genital tracts are protected by distinct epithelial cell layers, mucus, and by distinct innate and adaptive effector mechanisms. Specific immune cells in genital mucosae comprise intraepithelial T cells, macrophages, Langerhans cells (LCs), and submucosal DCs present in type II epithelia of the vaginal canals, which provide immune protection (Iwasaki 2010).

After infection, innate cells, including monocytes, neutrophils, NK cells, and plasmacytoid DCs (pDCs), are mobilized to the vaginal tissue. In the steady state, LCs in the epithelium and DCs in the submucosa are highly phagocytic and express high amounts of PRRs. 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. At a later time point, antigen-specific T and B cells enter the tissue to provide pathogen-specific immune defense. Due to the absence of inductive sites (MALT) in vaginal mucosa, priming of lymphocytes occurs exclusively in the draining lymph nodes including the common iliac, interiliac, external iliac, and inguinal femoral lymph nodes. Delivery of vaccines by genital routes is not very practical in human trials due to many disadvantages, comprising the cumbersome administration of a mucosal vaccine through the genital tract as well as the immunological features of the female reproductive tract due to hormonal fluctuations during the menstrual cycle (Kozlowski et al. 2002)

Oral route

The elicitation of immune responses in the intestinal mucosa by an orally administered antigen comprises its transportation by different pathways:

(1) Through M cells that are present in the follicle-associated epithelium of the PP or located in ILFs. Basolateral membrane of M cells is heavily invaginated while the apical one has little glycocalyx, presumably aiding antigen uptake, which is then captured by DCs, permitting their maturation and migration to the intrafollicular areas. M cells possess a high transcytotic capacity and are able to transport a broad range of materials. This pathway preferentially occurs for particulate antigens (Holmgren and Czerkinsky 2005; Neutra and Kozlowski 2006).

(2) Directly from the lumen by CX3CR1+ macrophages, (3) across epithelial cells, or (4) through epithelial tight junctions. The uptaken antigen can be transferred to CD103+ DC within the PP or in the lamina propria directly by these cells. The APCs process the antigen and migrate within the PP to the T cell areas and/or B cell follicles (inductive sites). T follicular helper (TFH) cells subsequently co-localize with B cells in the B cell follicle in close proximity to a follicular dendritic cell (FDC) network, and this allows the formation of a germinal center where the antigen-specific B cells undergo class-switching to IgA and somatic hypermutation to generate higher-affinity antibodies. Free antigen or antigen-loaded DCs from the PP or LP might gain access to draining lymph, with subsequent T cell recognition in the MLNs resulting in the induction of mucosal and systemic effector immune responses of T cells and B cell-producing IgA or IgG antibodies. The resulting IgA+ long-lived plasma cells and memory B cells generated within the germinal center leave the PP through the efferent lymph and migrate to the MLN and subsequently to the blood through the thoracic duct. Plasma cells home to bone marrow and to effector sites in the lamina propria of the small and large intestine. MLNs can act as a crossover point between the mucosal and systemic immunity and explain the induction of systemic immunity induced by intestinal antigens (Mowat 2003).

Table 2.1 Advantages and limitations of the distinct immunization routes

	Advantages	Limitations
Oral	Delivery: ingestion. Recipient-friendly approach	Requires mucosal adjuvant
	The delivery risk is minimal; no syringes or needles required	Required high antigen dose. Digestion in the gastrointestinal tract. Efficient uptake of particulate antigens
	Elicited responses: humoral and cell immune response. Mucosal IgA in large and small intestines, vagina, and salivary gland induce modest systemic antibody, and CTL responses	Can induce tolerance
	Extensive use for attenuated vaccines. Against rotavirus, *Vibrio cholera, Salmonella typhi,* and poliovirus	Limited clinical trials of subunit vaccines
	Is the safest route of vaccine delivery	
Nasal	The delivery risk is minimal; no syringes or needles required	Requires delivery devices. Requires full cooperation of the vaccinee
	Efficient antigen transfer across nasal epithelium	Requires mucosal adjuvant
	Elicited responses: systemic antibody and mucosal IgA in large intestine, vagina, and nasal cavity; CTL responses	Requires medium antigen dose
		Can induce tolerance
		Limited number of clinical trials. Against influenza
		Evidence of antigen transfer to neuronal tissue via olfactory bulb in mice. Clinical studies indicate that Bell's palsy is caused by influenza nasal vaccine that contains the native form of heat-labile *Escherichia coli*
Parenteral	Requires low antigen dose	Delivery: injection Requires medically trained personnel
	Potent systemic antibody and T cell	Possible transmission of infection by contaminated needles and syringes
	Extensive clinical use in many viral, bacterial, and parasitic diseases	Alum most widely used as adjuvant, but a variety of systems are effective
	Elicited responses: no major problems with subunit vaccines	Null response in mucosa
		Mild-to-serious side effects with killed or attenuated vaccines

Oral delivery of non-living vaccines has proved to be extremely challenging, owing to poor stability of proteins, peptides, and DNA in the acidic and enzyme-rich environments of the gastrointestinal tract. Several strategies, including the use of biodegradable polymeric particles and liposomes, had been adopted to protect antigens in the gastrointestinal tract. In addition, strong adjuvants, for example, enterotoxins

such as cholera toxin (CT) and the heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (LT), have been successfully used for the oral immunization of test animals. However, toxicity of these enterotoxins limits their applications in humans. To alleviate the toxicity issues, mutants and subunits of LT and CT have been used as adjuvants in many studies of oral immunization in animals with some promising perspectives (Box 2.4; Lycke 2012; Martin et al. 2000). Table 2.2 presents an overview of adjuvants and delivery vehicles developed for mucosal immunization.

Box 2.4 Adjuvants and Antigen-Delivery Systems

Intestinal immune system is tightly regulated and polarizes the immune response mainly to tolerogenic responses; thus, the development of new strategies for the enhancement of optimal immune response is urgently needed. Strong adjuvants such as bacterial enterotoxins (CT or LT) have been successfully used for oral immunization in mice. Recently, a rice-based vaccine that expressed CTB subunit has proved to serve as an effective long-term cold chain-free oral vaccine that induces CTB-specific sIgA-mediated long-standing protection against *V. cholerae* or LT-ETEC-induced diarrhea. As CTB lacks enzymatic toxic activity, this approach may overcome the limitations presented by CT or LT, which limit clinical uses (Holmgren and Czerkinsky 2005; Lycke 2012; Lawson et al. 2012).

Lectins possess the ability to activate the immune system, and this characteristic may also be exploited for oral immunization, since enhanced intestinal absorption by attaching to M cells in PP can be achieved. Plant lectins have demonstrated to be strong mucosal immunogens, stimulating systemic and mucosal antibody responses after oral or intranasal delivery (Lavelle et al. 2000; Rosales-Mendoza and Salazar-González 2014).

The formulation of antigens in various particulate delivery systems for mucosal administration may be advantageous in the following ways: (1) protects the antigen from degradative mucosal enzymes, (2) facilitates the preferential uptake of encapsulated antigen by M cells, (3) sustains the release of antigen to increase the presentation time of antigen to APCs, (4) allows for co-presentation of antigen and adjuvant to APCs, and (5) allows for the induction of cell-mediated immune response by modifying presentation of antigen to APCs. Therefore, rational antigen selection, adjuvants to angle-protective immune responses, efficient vectors to target APCs, and appropriate administration routes are key aspects to take into consideration in the development of efficient mucosal vaccines (Sharma and Hinds 2012; Valiante 2003; Pavot et al. 2012).

Typically, the doses that are required to elicit immune responses by the oral route are substantially higher, by up to 100-fold, than those requiring for parenteral formulations. This raises the crucial issue of the cost of immunization.

Table 2.2 Comprehensive list of adjuvants and delivery vehicles used in mucosal immunization approaches

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
Cholera toxin	Oral Nasal Vaginal Rectal	IgA, IgG1, IgE	Th2, CD8	Enhancement of antigen presentation by APCs	IL-4, IL-5, IL-6, IL-10	Enterotoxins composed of one active (A) and five binding (B) subunits	Lead to enhanced uptake of Antigens Enhance antigen presentation Promote isotype differentiation in B cells leading to increased IgA production	High toxicity Affinity to central nervous system Increase permeability of the intestinal epithelium	Chadwick et al. 2010; Holmgren et al. 2003; Freytag and Clements 2005; Yamamoto et al. 1997
E. coli heat-labile enterotoxin	Oral Nasal Vaginal Rectal	IgG1, IgG2, IgA	Th1/Th2, CD8	Enhancement of antigen presentation by APCs	IL-8, IL-10, IL-1(α,β), IL-6	ADP-ribosylating activity Binds to GM1 gangliosides expressed on the surface of many cell types			
Cry 1Ac Protoxin	Oral Intranasal intraperitoneal	Preferentially IgG1 IgA IgG2a		Increment of co-stimulatory molecules, CD80 and CD86, on APCs	IL-6 TNF-a MCP-1	Crystal protein 130 kDa From Bacillus thuringiensis	Non-toxic for mammalian Easy and low cost of production Friendly administration Induce mucosal and systemic responses protection against parasite infections	Mechanism of action has not been elucidated completely	Moreno-Fierros et al. 2013; Rojas-Hernández et al 2004
Virus-like particles	Oral Nasal Vaginal	Both serum IgG and IgA	Th1/Th2	Multiple mechanisms	Not determined	Plasmid DNA Proteins Peptides	Lacks viral genes Highly immunogenic High rate of uptake Undergoes self-assembly	Formulated by recombinant technology	Aguilar and Rodríguez 2007:Sharma et al. 2009; Holmgren et al. 2003; Reed et al. 2009; Eriksson and Holmgren 2002; Takamura et al. 2004; Ludwig and Wagner 2007

Table 2.2 (continued)

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
Protollin (LPS)	Nasal	Serum IgG, IgA	Th1/Th2	TLR4, TLR2	IFN-γ, MIP-3α, IL-18	Inflammatory responses by LPS-binding proteins LBP, CD14, MD-2, and TLR4	Release of inflammatory cytokines and upregulation of costimulatory molecules on antigen presenting cells	Severe toxicity in mammals	Chabot et al. 2005, 2007
CpG	Oral Nasal Vaginal Rectal	IgG2a, IgA	Th1/Th2, CD8	TLR9	IL-6, IL-12, IL-8 RANTES, MIP-1α MIP-1β, TNF-α, IFN-γ	Small oligodeoxynucleotides (ODN) Innate and adaptive immune responses	CpG ODN also directly activates monocytes, macrophages and DCs directly and should be superior adjuvant for intracellular pathogens	Suboptimal in vivo stability Toxicity Unfavorable pharmacokinetic/biodistribution Lack of specificity for target cells Requirement for intracellular uptake	Holmgren et al. 2003; Vajdy et al. 2004; McCluskie et al. 2000
Flagellin	Nasal	IgA, IgG	Th1/Th2	NLRC4, TLR5 and uses only MyD88 as the cytoplasmic adaptor	TNF-α, IFN-γ, MIP-2, IL-6	Flagellin is the only cognate ligand reported so far for TLR5	One of the very limited number of TLR ligand that could be engineered genetically Flagellin is a highly expressed and stable bacterial protein As a mucosal adjuvant, flagellin is almost as potent as CT or LT while it does not accumulate in olfactory nerve and bulb	Must await to determine whether recombinant flagellin-based vaccines trigger adverse events, such as a systemic cytokine storm or intense local inflammation at the site of immunization, which would limit their use	Coffman et al. 2010; Reed et al. 2009; Samatey et al. 2001; Weimer at al. 2009

Table 2.2 (continued)

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
MPL	Oral Nasal Vaginal Rectal		Th1/Th17	TLR4, TLR2	IL-1, IL-17, IFN-γ	Monophosphoryl lipid A bacterially derived product	Retains much of the immunostimulatory properties of the parent lipopolysaccharide without the inherent toxicity Mediate specific cellular immunity and enhanced levels of complementing antibodies Induce mucosal and systemic responses	Has been used extensively as an adjuvant for parenteral vaccines	Reed et al. 2009; Freytag and Clements 2004
QS21	Oral Nasal	IgG2a, IgG2b, IgG1, IgE	Th1		IL-4, IL-5, IL-6, IL-10	Extracted from the bark of *Quillaja saponaria*	Exhibit a remarkable ability to augment clinically significant responses to vaccine antigens targeting a wide landscape of diseases and degenerative disorders	Semi-purified extract consists of a mixture of triterpene saponins	Reed et al. 2009; Boyaka et al. 2001; Duthie et al. 2011; Chea et al. 2012
Chitosan/chitin	Oral Nasal	Serum IgG, sIgA	Antigen-dependent response	Electrostatic interaction with mucus and cell surfaces	IL-1β, IL-18	Non-toxic, biocompatible, and biodegradable It is converted from chitin by deacetylation	Stimulate macrophages by interacting with receptors Macrophages produce cytokines and other compounds that confer non-specific host resistance against bacterial and viral infections, and anti-tumor activity Chitin is a strong Th1 adjuvant Anti-allergic properties	Limited perception of the importance of the chemical/biochemical characteristics of the isolated chitin or chitosan for the replication of experiments and optimization of results	Reed et al. 2009; Li et al. 2013

Table 2.2 (continued)

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
Antigen delivery systems									
PLGA microparticles	Oral Nasal Vaginal	Antigen-dependent antibody	Antigen-dependent response	Mechanism undefined	Depends on immunostimulant	PLGA Plasmid DNA Protein Peptide Low-molecular-weight molecules Low loading efficiency	Controlled release Sensitive to environment Stable microenvironment Biocompatible	Degradation of antigen during encapsulation	Aguilar and Rodriguez 2007; Yih and Al-Fandi 2006; Mallapragada and Narasimhan 2008; Sinha and Trehan 2003
4a. PLA						Plasmid DNA Protein Peptide Lipophilic compound	Controlled release Surface easily modified	Low loading efficiency Degradation of antigen during encapsulation Cytotoxicity Reactogenicity	Sharma et al. 2009; Sinha and Trehan 2003; Lassalle and Ferreira 2007; Ogay et al. 2006
4b. PEI Plasmid						Plasmid DNA	Efficiently transfected	Cytotoxicity	Ogay et al. 2006; Forrest et al. 2003; Wong et al. 2006
Emulsions									
Water-in-oil emulsion									Chadwick et al. 2010 Joffret et al. 1990;
Oil-in-water emulsion						Water-insoluble drugs Water-soluble drugs Proteins DNA	Easy surface modification Synthesized from non-toxic material Dual function Wide range of antigen encapsulation	Low antigen loading Low stability	Alving 1991; Aguilar and Rodriguez 2007; Drummond et al. 2000; Sharma et al. 2009; Kersten and Crommelin 2003;
Liposomes						DNA cytotoxic agents Proteins	Efficient endocytic release	Intramembrane repulsion	Yih and Al-Fandi 2006 Drummond et al. 2000; Karmali and Chaudhuri 2007; Yih and Al-Fandi 2006

Table 2.2 (continued)

Adjuvant	Administration route	Antibody	Cellular responses	Proposed mechanism	Soluble factors	Characteristics	Advantages	Disadvantages	References
pH-sensitive liposomes						DNA siRNA	Controlled release of antigen	Non-specific interactions	Sato et al. 2007; Drummond et al. 2000; Drulis-Kawa and Dorotkiewicz-Jach 2010; Karmali and Chaudhuri 2007
Cationic liposomes	Oral Nasal Vaginal	IgG1, IgG2a	Th1/Th2, CD8		Not determined	Composed of antigen, cholesterol, phospholipid and saponins	Antigens evoke protective responses when prepared into ISCOMs	High production cost and low antigen binding	Medzhitov 2001; Reed et al. 2009
ISCOM	Oral Nasal Vaginal	IgG1, IgG2a	Th1/Th2, CD8	Saponin	Not determined	Particulate antigen delivery systems composed of antigen, cholesterol, phospholipid and saponins	Induce strong antigen-specific cellular or humoral immune responses to a broad range of antigens of viral, bacterial, and parasite origin or tumors	High production cost	Medzhitov 2001; Reed et al. 2009; Hong-Xiang et al. 2009
Plants	Oral	IgG IgA	Th1	Gradual release of antigen in the gastrointestinal tract	Depending on the antigen	Plant cells serve as biofactories and delivery vehicles Concept has been proven for a wide variety of human infectious and autoimmune diseases	Low cost Easy storage Absence of mammalian pathogens Delay antigen degradation Possible adjuvant effect mediated by plant metabolites	Variability in expression levels Characterization in each lot of antigen Possible induction of immune response against plant proteins	Govea-Alonso et al. 2013; Yusibov et al. 2011; Kostrzak et al. 2009

Mucus provides a highly viscous and heterogeneous microenvironment that presents a significant barrier not only for pathogen entry but also to mucosal vaccine delivery. Therefore, in order to be effective, mucosal vaccines must prevent inactivation of both the antigen and the adjuvant by the harsh mucosal environment and deliver the vaccine across mucosal barriers to target mucosal tissues and immune cells. The pore size of mucus has been estimated to range 50–1,800 nm. Surface modification of drug-delivery vehicles has proven to be a promising approach to increase both mucoadhesion and mucus penetration. Several natural materials such as chitosan, alginate, and derivatives of cellulose show strong mucoadhesive properties owing to the presence of numerous hydrogen bond-forming groups. The concepts of mucus penetration and mucus adhesion will have a significant role in achieving effective transport of mucosally administered vaccines (Woodrow et al. 2012).

The induction of mucosal immune responses against foreign antigens, microorganisms, and vaccines requires the presence of organized lymphoid tissue, either within the mucosa or in draining lymph nodes. Soluble, non-adherent antigens are taken up at low levels, if at all, and such antigens generally induce tolerance in the intestine. In general, mucosal vaccines are likely to be most effective when they mimic successful mucosal pathogens in the following key respects: They are ideally multimeric and/or particulate, adhere to mucosal surfaces (or even better, adhere selectively to M cells), efficiently stimulate innate responses, and evoke adaptive responses that lead to immunoprotection against the target pathogen (Neutra and Kozlowski 2006). Particulate vaccines have theoretical advantages for mucosal delivery because M cells are known to uptake efficiently microparticles with a diameter of up to 1 μm. Encapsulation of antigens in polymer-based particles can be a promising tool for delivery of vaccines to mucosal sites. However, without proper targeting, these carriers may not be successfully internalized, processed, and presented in a way to direct an immunological response. Targeting APCs, specifically DCs, constitutes another strategy. Most examples of DC-targeting strategies employ the well-characterized DC receptor DC205, DC-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), or mannose receptor. Mucosal epithelial cells represent another opportunity for targeting vaccines. Potential targets to address this objective are the epithelial markers FcRn and galactosyl ceramide (Woodrow et al. 2012). Another method that has been employed to favor adhesion between epithelium and the vaccine delivery vehicle consists of using high-affinity targeting ligands against M cells, but only few M cell receptors had been identified. As M cells tend to exhibit unique glycosylation patterns, lectins such as *Ulex europeus* agglutinin 1 (UEA-1), which binds alpha-1-fucose, has been the most widely investigated M cell-targeting molecules in mice (Pavot et al. 2012).

Accordingly, the effectiveness of live pathogens and effective oral vaccines such as the live poliovirus and live attenuated *S. typhi* vaccines is partly a result of their adaptation to survive in luminal environments, due to which they can efficiently invade organized lymphoid tissues in the intestines. Non-living macromolecules, protein-subunit antigens, and non-microbial particles generally evoke weak immune responses when applied mucosally, and thus the use of adjuvants is required in order to alert the mucosa and activate innate signaling pathways in epithelial cells

or in the underlying APCs. However, the major limitation of using live vaccines and adjuvants are associated with toxicity risks (Pavot et al. 2012).

Under this outlook, it is clear that efforts to overcome obstacles in the development of effective mucosal vaccines are mainly directed towards finding more efficient means of delivering appropriate antigens to the mucosal immune system, and towards discovering effective, safe mucosal adjuvants capable of providing protective immunity against infectious agents.

Vaccines based on live attenuated viruses or microbes that have been inactivated by heat or chemicals comprise the majority of licensed vaccines used for the prevention of infectious diseases. To date, these constitute the only vaccines approved for mucosal delivery and the only ones whose efficacy is correlated with effector mucosal immune response (Woodrow et al. 2012). The oral polio vaccine is a live attenuated vaccine that produces serum antibodies as well as local sIgA in the intestinal mucosa, which confers protection from virus entry and multiplication. Other live attenuated vaccines administered via the oral route are licensed for enteric infections such as cholera, typhoid, and rotavirus. The success of live attenuated or inactivated vaccines is attributed to the presentation of multiple immunogens and enhanced second signals that combine and elicit strong antibody responses and long-term memory. However, not all viruses can be attenuated, and the risk of reversion can compromise safety, especially for viruses with ill-defined attenuation. Although inactivation of viruses and bacteria is a more generalized approach and these vaccines are much safer, inactivated vaccines can exhibit loss of antigens or pathogen-associated molecular patterns (PAMPs). This loss results in rapid waning of protective immunity and causes the inactivated vaccines to be less effective than live attenuated vaccines (Woodrow et al. 2012).

Subunit vaccines and conjugated vaccines are a second largest category of licensed prophylactic vaccines. These vaccines are based on pathogen-specific proteins or polysaccharides conjugated to proteins or peptides. Subunit and conjugate vaccines as well as toxoid vaccines are administered primarily by subcutaneous or intramuscular routes and not mucosally. One notable exception is a vaccine against cholera toxin B subunit and the inactivated strain of *V. cholerae* O1. Oral but not parenteral immunization, with inactivated whole-cell cholera bacteria together with cholera toxin B subunit, protects against cholera colonization and toxin binding. This vaccine induces protection-specific mucosal antibodies and provides long-lasting intestinal immunological memory. However, no other examples of successful licensed subunit vaccines that are administered by mucosal immunization and provide protection are available (Woodrow et al. 2012). The use of living microorganism for the delivery of antigens has shown to induce mucosal immune responses at the gastrointestinal and the systemic levels (Neutra and Kozlowski 2006). Therefore, oral delivery of antigens in attenuated bacterial strains is an alternative solution to antigen protection, but raises safety concerns over the delivery vehicles.

Another approach consists on the use of plant-based vaccines, which provide a means to deliver large amounts of a designated antigen in an encapsulated form. Plants have been used to express a wide range of recombinant proteins, including diagnostic proteins, industrial enzymes, and enzymes used in the production

of pharmaceuticals, food additives, therapeutic proteins, antibodies, and vaccine antigens (Daniell et al. 2009). Levels of expression achieved thus far indicate the long-term economic viability of plant-based systems for recombinant protein production. In the case of subunit vaccines, large-scale antigen production in plant systems should be sufficiently inexpensive to allow for delivery of the necessary high dosages anticipated for oral administration. Production of antigens in plant material has the added advantage of encapsulation in the expression host since antigens are naturally encapsulated in the tissue used for recombinant protein production. This encapsulation appears to guard against rapid and complete degradation of orally administered recombinant proteins. Thus, there is the potential for antigen to be gradually released into the gastrointestinal tract as long as plant cells are digested. This should allow for an increased proportion of orally administered antigens to reach effector sites which line the gastrointestinal tract (Pavot et al. 2012; Rosales-Mendoza and Salazar-González 2014). This approach has yielded encouraging results in animals and humans, although the safety of transgenic plans needs to be further evaluated (Mitragotri 2005). Although in its infancy, oral immunization by means of plant-based vaccines augurs a potential source of novel vaccines. Chapter 13 provides relevant "Plant-Based Vaccines as a Global Vaccination Approach: Current Perspectives" perspectives on future research activity that is considered critical to favor the advancement of this technology.

Acknowledgments This work was supported by the following grants: PAPIIT IN219013 and CONACYT 177612.

References

Aguilar JC, Rodríguez EG (2007) Vaccine adjuvants revisited. Vaccine 25:3752–3762

Alving CR (1991) Liposomes as carriers of antigens and adjuvants. J Immunol Methods 140(1):1–13

Artis D (2008) Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol 8:411–420

Boyaka PN, Marinaro M, Jackson RJ, van Ginkel FW, Cormet-Boyaka E, Kirk KL, Kensil CR, McGhee JR (2001) Oral QS-21 requires early IL-4 help for induction of mucosal and systemic immunity. J Immunol 166:2283–2290

Brandtzaeg P, Farstad IN, Haraldsen G (1999) Regional specialization in the mucosal immune system: primed cells do not always home along the same track. Immunol Today 20:267–277

Chabot S, Brewer A, Lowell G, Plante M, Cyr S, Burt DS, Ward BJ (2005) A novel intranasal Protollin-based measles vaccine induces mucosal and systemic neutralizing antibody responses and cell-mediated immunity in mice. Vaccine 23:1374–1383

Chabot SM, Chernin TS, Shawi M, Wagner J, Farrant S, Burt DS, Cyr S, Neutra MR (2007) TLR2 activation by proteosomes promotes uptake of particulate vaccines at mucosal surfaces. Vaccine 25:5348–5358

Chadwick S, Kriegel C, Amiji M (2010) Nanotechnology solutions for mucosal immunization. Adv Drug Deliv Rev 62:394–407

Chea EK, Fernández-Tejada A, Damani P, Adams MM, Gardner JR, Livingston PO, Ragupathi G, Gin DY (2012) Synthesis and preclinical evaluation of QS-21 variants leading to simplified vaccine adjuvants and mechanistic probes. J Am Chem Soc 134:13448–13457

Cheroutre H, Lambolez F, Mucida D (2011) The light and dark sides of intestinal intraepithelial lymphocytes. Nat Rev Immunol 11:445–456

Coffman RL, Sher A, Seder RA (2010) Vaccine adjuvants: putting innate immunity to work. Immunity 33:492–503
Coombes JL, Powrie F (2008) Dendritic cells in intestinal immune regulation. Nat Rev Immunol 8:435–446
Daniell H, Singh ND, Mason H, Streatfield SJ (2009) Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci 14:669–679
Drulis-Kawa Z, Dorotkiewicz-Jach A (2010) Liposomes as delivery systems for antibiotics. Int J Pharm 387:187–198
Drummond DC, Zignani M, Leroux J (2000) Current status of pH-sensitive liposomes in drug delivery. Prog Lipid Res 39:409–460
Duthie MS, Windish HP, Fox CB, Reed SG (2011) Use of defined TLR ligands as adjuvants within human vaccines. Immunol Rev 239:178–196
Eriksson K, Holmgren J (2002) Recent advances in mucosal vaccines and adjuvants. Curr Opin Immunol 14:666–672
Forrest ML, Koerber JT, Pack DW (2003) A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery. Bioconjug Chem 14:934–940
Freytag LC, Clements JD (2005) Mucosal adjuvants. Vaccine 23:1804–1813
Govea-Alonso DO, Rubio-Infante N, García-Hernández AL, Varona-Santos JT, Korban SS, Moreno-Fierros L, Rosales-Mendoza S (2013) Immunogenic properties of a lettuce-derived C4(V3)6 multiepitopic HIV protein. Planta 238(4):785–92. doi: 10.1007/s00425-013-1932-y. Epub 2013 Jul 30.
Geissmann F, Gordon S, Hume DA, Mowat AM, Randolph GJ (2010) Unravelling mononuclear phagocyte heterogeneity. Nat Rev Immunol 10:453–460
Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11:S45–S53
Holmgren J, Czerkinsky C, Eriksson K, Mharandi A (2003) Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. Vaccine 21:S89–S95
Holt PG, Strickland DH, Wikström ME, Jahnsen FL (2008) Regulation of immunological homeostasis in the respiratory tract. Nat Rev Immunol 8:142–152
Hong-Xiang S, Yong X, Yi-Ping Y (2009) ISCOMs and ISCOMATRIX. Vaccine 27:4388–4401
Iwasaki A (2010) Antiviral immune responses in the genital tract: clues for vaccines. Nat Rev Immunol 10:699–711
Joffret ML, Morgeaux S, Laclerc C, Oth D, Zanetti C, Sureau P, Perrin P (1990) Enhancement of interleukin-2 activity by liposomes. Vaccine 8:385–389
Karmali PP, Chaudhuri A (2007) Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises. Med Res Rev 27:696–722
Kersten GF, Crommelin DJ (2003) Liposomes and ISCOMs. Vaccine 21:915–920
Kostrzak A, Cervantes Gonzalez M, Guetard D, Nagaraju DB, Wain-Hobson S, Tepfer D, Pniewski T, Sala M (2009) Oral administration of low doses of plant-based HBsAg induced antigen-specific IgAs and IgGs in mice, without increasing levels of regulatory T cells. Vaccine 27:4798–4807
Kozlowski PA, Williams SB, Lynch RM, Flanigan TP, Patterson RR, Cu-Uvin S, Neutra MR (2002) Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. J Immunol 169:566–574
Kunkel EJ, Butcher EC (2002) Chemokines and the tissue-specific migration of lymphocytes. Immunity 16:1–4
Lassalle V, Ferreira ML (2007) PLA nano- and microparticles for drug delivery: an overview of the methods of preparation. Macromol Biosci 7:767–783
Lavelle EC, Grant G, Pusztai A, Pfüller U, O'Hagan DT (2000) Mucosal immunogenicity of plant lectins in mice. Immunology 99:30–37
Lawson LB, Norton EB, Clements JD (2012) Defending the mucosa: adjuvant and carrier formulations for mucosal immunity. Vaccine 30:142–154
Li X, Min M, Du N, Gu Y, Hode T, Naylor M, Chen D, Nordquist RE, Chen WR (2013). Chitin, chitosan, and glycated chitosan regulate immune responses: the novel adjuvants for cancer vaccine. Clin Dev Immunol 2013:387023

Ludwig C, Wagner R (2007) Virus-like particles—universal molecular toolboxes. Curr Opin Biotechnol 18:537–545
Lycke N (2012) Recent progress in mucosal vaccine development: potential and limitations. Nat Rev Immunol 12:592–605
Mallapragada SK, Narasimhan B (2008) Immunomodulatory biomaterials. Int J Pharm 364:265–271
Manicassamy S, Pulendran B (2009) Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. Semin Immunol 21:22–27
Mantis NJ, Rol N, Corthésy B (2011) Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. Mucosal Immunol 4:603–611
Martin M, Metzger DJ, Michalek SM, Connell TD, Russell MW (2000) Comparative analysis of the mucosal adjuvanticity of the type II heat-labile enterotoxins LT-IIa and LT-IIb. Infect Immun 68:281–287
McCluskie MJ, Weeratna RD, Krieg AM, Davis HL (2000) CpG DNA is an effective oral adjuvant to protein antigens in mice. Vaccine 19:950–957
Medzhitov R (2001) Toll-like receptors and innate immunity. Nat Rev Immunol 1:135–145
Mitragotri S (2005) Immunization without needles. Nat Rev Immunol 5:905–916
Moreno-Fierros L, García-Hernández AL, Ilhuicatzi-Alvarado D, Rivera-Santiago L, Torres-Martínez M, Rubio-Infante N, Legorreta-Herrera M (2013) Cry1Ac protoxin from Bacillus thuringiensis promotes macrophage activation by upregulating CD80 and CD86 and by inducing IL-6, MCP-1 and TNF-α cytokines. Int Immunopharmacol 17(4):1051–66. doi: 10.1016/j.intimp.2013.10.005. Epub 2013
Mowat AM (2003) Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol 3:331–341
Neutra MR, Kozlowski PA (2006) Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 6:148–158
Ogay ID, Lihoradova OA, Azimova ShS, Abdukarimov AA, Slack JM, Lynn DE (2006) Transfection of insect cell lines using polyethylenimine. Cytotechnology 51:89–98
Pabst O, Mowat AM (2012) Oral tolerance to food protein. Mucosal Immunol 5:232–239
Pavot V, Rochereau N, Genin C, Verrier B, Paul S (2012) New insights in mucosal vaccine development. Vaccine 30:142–154
Rappuoli R, Mandl CW, Black S, De Gregorio E (2011) Vaccines for the twenty-first century society. Nat Rev Immunol 11:865–872. doi:10.1038/nri3085
Reed SG, Bertholet S, Coler RN, Friede M (2009) New horizons in adjuvants for vaccine development. Trends Immunol 30:23–32
Rescigno M (2011) The intestinal epithelial barrier in the control of homeostasis and immunity. Trends Immunol 32:256–264
Rojas-Hernández S, Rodríguez-Monroy MA, López-Revilla R, Reséndiz-Albor AA, Moreno-Fierros L (2004) Intranasal coadministration of the Cry1Ac protoxin with amoebal lysates increases protection against *Naegleria fowleri* meningoencephalitis. Infect Immun 72:4368–4375
Rosales-Mendoza S, Salazar-González JA (2014) Immunological aspects of using plant cells as delivery vehicles for oral vaccines. Expert Rev Vaccines 13:737–749
Samatey FA, Imada K, Nagashima S, Vonderviszt F, Kumasaka T, Yamamoto M, Namba K (2001) Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling. Nature 410:331–337
Sato A, Takagi M, Shimamoto A, Kawakami S, Hashida M (2007) Small interfering RNA delivery to the liver by intravenous administration of galactosylated cationic liposomes in mice. Biomaterials 28:1434–1442
Scott CL, Aumeunier AM, Mowat AM (2011) Intestinal CD103 + dendritic cells: master regulators of tolerance? Trends Immunol 32:412–419
Shale M, Schiering C, Powrie F (2013) CD4(+) T-cell subsets in intestinal inflammation. Immunol Rev 252:164–182
Sharma S, Hinds LA (2012) Formulation and delivery of vaccines: ongoing challenges for animal management. J Pharm Bioallied Sci 4:258–266

Sharma S, Mukkur TK, Benson HA, Chen Y (2009) Pharmaceutical aspects of intranasal delivery of vaccines using particulate systems. J Pharm Sci 98:812–843

Sinha VR, Trehan A (2003) Biodegradable microspheres for protein delivery. J Control Release 90:261–280

Strugnell RA, Wijburg OL (2010) The role of secretory antibodies in infection immunity. Nat Rev Microbiol 8:656–667

Takamura S, Niikura M, Li TC, Takeda N, Kusagawa S, Takebe Y, Miyamura T, Yasutomi Y (2004) DNA vaccine-encapsulated virus-like particles derived from an orally transmissible virus stimulate mucosal and systemic immune responses by oral administration. Gene Ther 11:628–635

Vajdy M, Srivastava I, Polo J, Donnelly J, O'Hagan D, Singh M (2004) Mucosal adjuvants and delivery systems for protein-, DNA- and RNA-based vaccines. Immunol Cell Biol 82:617–627

Valiante NM (2003) Recent advances in the discovery and delivery of vaccine adjuvants. Nat Rev Drug Discov 2:727–735

Weimer ET, Ervin SE, Wozniak DJ, Mizel SB (2009) Immunization of young African green monkeys with OprF epitope 8-OprI-type A- and B-flagellin fusion proteins promotes the production of protective antibodies against nonmucoid *Pseudomonas aeruginosa*. Vaccine 27:6762–6769

Wong K, Sun G, Zhang X, Dai H, Liu Y, He C, Leong KW (2006) PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo. Bioconjug Chem 17:152–158

Woodrow KA, Bennett KM, Lo DD (2012) Mucosal vaccine design and delivery. Annu Rev Biomed Eng 14:17–46

Yamamoto S, Kiyono H, Yamamoto M, Imaoka K, Fujihashi K, Van Ginkel FW, Noda M, Takeda Y, McGhee JR (1997) A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci U S A 94:5267–5272

Yih TC, Al-Fandi M (2006) Engineered nanoparticles as precise drug delivery systems. J Cell Biochem 97:1184–1190

Yuki Y, Kiyono H (2009) Mucosal vaccines: novel advances in technology and delivery. Expert Rev Vaccines 8:1083–1097

Yusibov V, Streatfield SJ, Kushnir N (2011) Clinical development of plant-produced recombinant pharmaceuticals: vaccines, antibodies and beyond. Hum Vaccin 7:313–321