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Chapter 3
Mucosal Vaccination: Opportunities and Challenges

Olga Borges and Gerrit Borchard

3.1 Introduction

Mucosal vaccination has been the common generic name attributed to the oral, intranasal, pulmonary, rectal, and vaginal routes of vaccine administration. Mucosal surfaces, with a combined surface area of about 400 m^2 [1], are undoubtedly the major site of entry for most pathogens. Therefore, these vulnerable surfaces are associated with a large and highly specialized innate and adaptive mucosal immune system that protects the surfaces and the body against potential destructive agents and harmless substances from the environment. In a healthy human adult, this local immune system contributes almost 80% of all immune cells [2]. These immune cells accumulate in a particular mucosa or circulate between various mucosa-associated lymphoid tissues (MALT), which together form the largest mammalian lymphoid organ system [1]. In theory, mucosal surfaces seem to be the more accessible lymphoid organ for the induction of an immune response such as that required for immunization. Nevertheless, one of the more important reasons for the development of mucosal vaccines is the increasing evidence that local mucosal immune responses are important for protection against disease, principally for diseases which start on mucosal surfaces such as the respiratory, gastrointestinal, or urogenital mucosae. On the other hand, mucosal immune responses are most efficiently induced by the administration of vaccines onto mucosal surfaces, while injected vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infection at mucosal surfaces. However, even

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with the many attractive features of mucosal vaccination described, it has often proven difficult in practice to stimulate strong sIgA immune responses and protection by mucosal antigen administration [2]. As a consequence, no more than half a dozen mucosal vaccines are currently approved for human use and no subunit vaccines are listed among those approved.

3.2 Anatomophysiology of the Mucosal Immune System

MALT includes the gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), nasopharynx-associated lymphoid tissue (NALT), the mammary and salivary glands, and the urogenital organs. The common mucosal immune system (CMIS) acts as an integrated pathway that establishes communication between the organized MALT (inductive sites) and the diffuse mucosal tissues (effector sites). However, there is some evidence supporting the theory that this CMIS is compartmentalized. For instance, stimulation at one mucosal site in MALT can induce an immune response at remote mucosal effector sites [3, 4]. However, the extent of the immune response at the effector sites depends on where the induction occurred. Holmgrenn and Czerkinsky recently summarized this phenomenon in this way: "Oral immunization may induce substantial antibody responses in the small intestine (strongest in the proximal segment), ascending colon and mammary and salivary glands and it is relatively inefficient at evoking an IgA antibody response in the distal segments of the large intestines, tonsils or female genital tract mucosa. Conversely, intranasal immunization in humans results in antibody responses in the upper airway and cervicovaginal mucosa, and regional secretions (saliva, nasal secretions) without inducing an immune response in the gut" [2]. Important evidences that may explain, at least in part, the dependence of the mucosal site where the IgA is generated on the route of antigen administration were recently summarized by Kiyono [5].

3.2.1 Gut-Associated Lymphoid Tissue

The GALT described elsewhere [1] lines the digestive system and has two organizational levels to its structure: one with little organization, characterized by loose clusters of lymphoid cells in the lamina propria of the intestinal villi, and the other with a high level of organization called Peyer's patches.

The so-called intraepithelial lymphocytes (IELs) can be found in the outer mucosal epithelial layer, and the majority of these cells are CD8+ T-lymphocytes. Due to its localization, it is thought that this population of T cells may function to encounter antigens that enter through the intestinal mucous epithelium. Under the epithelial layer is the lamina propria, which contains large numbers of B cells, plasma cells, activated T_H cells, and macrophages in loose clusters. It is interesting to note that in healthy children, histological sections of the lamina propria have revealed more than 15,000 lymphoid follicles in total (described in [1]).

Peyer's patches, located in the submucosal layer underneath the lamina propria, contain between 30 and 40 lymphoid follicles organized as macroscopic nodules or aggregates. In a similar way to what happens with lymphoid follicles in other sites, those from mature Peyer's patches can develop into secondary follicles with germinal centers, supported or connected by follicular dendritic cells.

Parafollicular T-lymphocyte zones located between the large B-cell follicles present a large number of high endothelium venules, allowing cellular migration and lymphocytes recirculation.

Between the follicle-associated epithelium (FAE) and the organized lymphoid follicle aggregates, there is a more diffuse area known as the subepithelial dome (SED).

The FAE is the name given to the mucous membrane overlying the organized lymphoid follicles. The FAE is a small region characterized by the presence of specialized flattened epithelial cells called M-cells. Together, the FAE, lymphoid follicles, and associated structures form the antigen sampling and inductive sites of the mucosal immune system [6].

The function and structural characteristics of microfold epithelial cells (M cells) have been described in several recent reviews [1, 6]. It has been widely accepted that M cells are probably playing a key role in mucosal infection and immunity. It is thought that the main role of M-cells is the sampling of antigens to transport them across mucosal epithelia to the underlying lymphoid tissues where protective immune responses are generated. In addition, M-cells are a common route for complex antigens and pathogen invasion, for example, several invasive *Salmonella* species, *Vibrio cholerae*, *Yersinia* species, *Escherichia coli* and the polio virus [6].

M-cells have been identified in the epithelia of a variety of mucosal tissues and within the FAE of a wide variety of animal species, including laboratory animals (mice, rats, rabbits), domestic pets, and man. In mice and men, M-cells reside in about 10% of the FAE in contrast with 50% in the rabbit. In the gut, M-cells are easily recognized by the lack of surface microvilli and the normal thick layer of mucus that characterizes the rest of the epithelial cells. Additionally, M-cells contain a deep invagination similar to a pocket in the basolateral cytoplasmic membrane that contains one or more lymphocytes and occasional macrophages [6].

3.2.2 Nasopharynx-Associated Lymphoid Tissue

In rodents, NALT is found on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate, and it is considered to be analogous to Waldeyer's ring in humans (pharyngeal lymphoid tissue that includes adenoid, tubal tonsil, palatine tonsil, lingual tonsil) [7]. In the rat, lymphoid aggregates are situated at the nasal entrance to the pharyngeal duct [8]. Detailed reviews of NALT and nasal vaccination can be found elsewhere [8–10]. NALT is a well-organized structure consisting of B- and T-cell-enriched areas which are covered by an epithelial layer containing

M-cells, the so-called FAE. The function of these antigen-sampling M cells seems to be similar to those found on the FAE of Peyer's patches [5]. Although NALT and Peyer's patches share certain similarities, they two differ markedly in morphology, lymphoid migration patterns, and the binding properties of the [high] endothelial venules [7]. Additionally, IELs and antigen-presenting cells including dendritic cells (DCs) and macrophages can also be found in NALT [11]. Therefore, according to Kiyono [5], NALT contains all of the lymphoid cells that are required for the induction and regulation of mucosal immune response to antigens delivered to the nasal cavity.

3.3 Immune Responses Initiated by MALT

MALT plays an important role in antigen sampling and generation of lymphocytes, including specific IgA effector B cells, memory B cells and T cells. This involves active lymphocyte proliferative activity, local production of cytokines, and continuous cellular trafficking [12]. Antigens from the lumen can be internalized by antigen-processing dendritic cells which move into the epithelium and then migrate back to local or distant organized tissues. In the intestinal and airway epithelia, mucosal epithelial cells are sealed by tight junctions; therefore, most of antigen (predominantly particulates) transport is carried out by the M cells. Luminal antigens are endocytosed into vesicles that are transported from the luminal membrane to the underlying M-cell pocket membrane. Vesicles and the pocket membrane experience fusion, and the antigens are delivered to the clusters of lymphocytes present within the pocket. It is not known whether M cells participate in antigen processing and presentation nor if they express MHC class II molecules [12, 13]. Simultaneously, it is believed that the intact antigens are processed by professional antigen-presenting cells (APCs) such as macrophages and dendritic cells, either in the epithelium or in the underlying dome region immediately below M-cells which is thus ideally located to sample transported antigens. Moreover, chemokines secreted by the FAE result in an additional attraction of DCs to the FAE, resulting in a high density of phagocytic cells at sites of entry of foreign antigens and pathogens [14]. Phenotypically immature DCs are subsequently moved to the T-cell areas, where they upregulate the expression of maturation markers and MHC molecules [14].

In the follicle, B cells undergo immunoglobulin class switching from expression of IgM to IgA under the influence of several local factors, including transforming growth factor (TGF-β), IL-10 and cellular signals delivered by dendritic cells and T cells [13]. Furthermore, it is thought that because dendritic cells are migratory cells, they can transport microbes to the mesenteric lymph nodes and to the spleen for the induction of systemic responses [15]. Therefore, these cells also possibly transport antigens, especially those sampled directly from the luminal contents.

The lymphocytes primed in the Peyer's patches move through the draining lymphatics to the mesenteric lymph nodes (MLN) where they can reside for an undefined period for further differentiation before they migrate again to the mucosa.

Peyer's patches contain all the cellular and microarchitectural environments (e.g., a B-cell follicle including germinal centers, a dendritic cell network, and an interfollicular T-cell area) needed for the generation of IgA-committed B cells [16]. Therefore, B cells primed in the Peyer's patches or in NALT and transported to the MLN migrate again to the diffuse mucosal effector tissues such as the lamina propria of the upper respiratory and intestinal tract where full maturation is achieved under the influence of IgA-enhancing cytokines IL-5, IL-6, and IL-10 and are transformed into immunoglobulin-secreting active plasma or blast cells [5, 16].

How the lymphocytes know where to return is an interesting and important aspect of the mucosal immune response. It seems to be well established already that following activation in organized mucosal lymphoid tissues, B- and T-cells are able to upregulate the expression of tissue-specific adhesion molecules and chemokine receptors that function as "homing receptors" to guide the lymphocytes back to the mucosa through the recognition of endothelial counter-receptors in the mucosal vasculature [14, 17, 18].

Although IgA constitutes only 10–15% of the total immunoglobulin in serum, it is the predominant immunoglobulin class in external secretions such as breast milk, saliva, tears, and mucus of the bronchial, genitourinary, and digestive tracts [1]. In humans, more IgA is produced than all other immunoglobulin isotypes combined, and IgA is concentrated over 1 mg/mL in secretions associated with the mucosal surfaces [14].

The secretory immunoglobulin A has several functions in mucosal defense as described elsewhere [2, 5, 14]. So-called immune exclusion is a mechanism that consists of the entrapment of antigens or microorganisms by the sIgA in mucus, preventing direct contact of the antigen with the mucosal surface [14, 19]. Additionally, specific sIgA might block or sterically hinder the microbial surface molecules that mediate epithelial attachment [20].

IgA on the mucosal surface and within the lamina propria is able to complex with food or environmental antigens. The resulting immune complexes may be destroyed locally or excreted through the overlying epithelium, thus preventing potentially antigenic materials from reaching the circulation where they may be able to induce IgE antibodies with subsequent development of food allergy. Therefore, IgA also serves as an immunological barrier to environment antigens.

3.4 Challenges in Oral and Nasal Vaccine Design

Vaccines administered through one of the mucosal surfaces encounter the same host mechanisms as harmless antigens, such as food proteins and commensal bacteria or the same defense barriers as do microbial pathogens and other foreign macromolecules. Therefore, after mucosal administration, vaccines can be diluted in mucosal secretions, detained in mucus gels, attacked by proteases and nucleases and barred by epithelial barriers. Therefore, it is estimated that large doses of antigen would be required. Moreover, soluble non-adherent antigens are taken up at low levels if at

all, and in the intestine, such antigens can induce immune tolerance [21] or simply be ignored be the mucosal immune system [22].

3.4.1 Active Components in Gastrointestinal Luminal Fluids

Besides the barrier function of the mucus covering all mucosal surfaces, the gastrointestinal system has additional important specificities that constitute a barrier to vaccine (attenuated or killed bacteria, antigen proteins, peptides) administration, the gastric and intestinal fluids. The mucosal layer of stomach is an epithelium covered with tiny gastric pits that are entrances to millions of gastric glands. These glands contain cells that secrete some of the products needed to digest food. The secretion of the gastric juice is stimulated by signals from the stretch receptors that are activated by food entrance into stomach. The most important components of gastric juice are pepsinogen, the precursor for the digestive enzyme pepsin, hydrochloric acid (HCl), and lubricanting mucus. It is long known that pepsinogen is converted to the digestive enzyme pepsin by the highly acid conditions of the stomach. HCl causes proteins in the digestive contents to unfold, exposing their peptide linkages to hydrolysis by pepsin. The HCl also kills most of the bacteria that reach the stomach and stops the action of the salivary amylase. In duodenum, chime or other foreign compounds (vaccines, proteins, bacteria, virus, etc.) contact with a fluid that result from the contribution of gastric, pancreatic and liver secretions. Therefore, the luminal fluids of the first segment of the intestine have high concentrations of pancreatic enzymes, which include proteases (active forms are trypsin, carboxypeptidase), an amylase, nucleases, lipases, and bicarbonate ions ($H_2CO_3^-$). The liver contributes also with $H_2CO_3^-$ and bile (bile salts, cholesterol, and bilirubin) which are important for the emulsion of the food fats.

Brush-border epithelial cells on the villi of small intestine secrete water and mucus into the intestinal contents. These cells also produce enzymes (some examples are: disaccharidases such as lactase, aminopeptidase, nucleases, nucleotidases, nucleosidases) that complete the digestion of carbohydrates (disaccharides), proteins (large peptides, dipeptides) and nucleic acids (nucleotides). A large amount of other proteases not mentioned in this text are secreted into luminal fluids. All constitute an enzymatic barrier for peptide and protein antigens GI delivery (reviewed in [23]).

3.4.2 Physical Epithelial Barriers

Mucosal epithelial cells are tightly linked via intracellular junctions that form a continuous barrier which is resistant to microbial passage, the epithelial tight junctions.

The other barrier to infection is the cell surface mucin barrier and the glycocalyx.

3.4.2.1 Tight Junctions

Tight junctions are a form of cell–cell adhesion in epithelial and endothelial cellular sheets. They are responsible for intercellular sealing. Therefore they act as a primary barrier or "gate" to the diffusion of solutes or larger particles, including pathogens, through the intercellular space. But many physiological situations require that various materials are selectively transported across cellular sheets, and this occurs either by transcellular transport through the cell or by paracellular flux through tight junctions. So, tight junctions are not simply impermeable barriers: they show ion as well as size selectivity, and vary in tightness depending on the cell type. In addition to the "barrier function," tight junctions are thought to function as a "fence" [24] to prevent diffusion or intermixing of plasma membrane components between the apical and basolateral domains. It has been demonstrated that some human pathogens are able to invade the body through epithelial cells. It was demonstrated that in some cases they interfere with epithelial polarity to enhance binding to the apical surface, enter into cells, and/or cross the mucosal barrier [25]. On other cases it was demonstrated that dendritic cells (DCs) open the tight junctions between epithelial cells, send dendrites outside the epithelium and directly sample bacteria. In addition, because DCs express tight-junction proteins such as occludin, claudin 1, and zonula occludens 1, the integrity of the epithelial barrier is preserved [26]. On the other hand, tight junctions can be opened using diverse absorption enhancers. Among those compounds, chitosan has been intensely studied [27].

3.4.2.2 Extracellular Mucus Barrier

Epithelial layers in the body are protected from pathogens and similar stresses by mucus. However, successful enteric pathogens have created strategies to circumvent these barriers. Early investigations of diffusion through mucus gels demonstrated that small molecules can readily diffuse through mucus whereas mucus is an impermeable elastic barrier to bacterium-sized particles. This appears rational, since the end products of digestion, such as monosaccharides and disaccharides, or small peptides, could penetrate the mucous layers to reach the enterocytes and to undergo subsequent absorption. More recent work clearly demonstrates that virus-sized particles can readily diffuse through mucus gels [28]. Therefore, detailed knowledge of mucin dynamics is required to understand the interaction of the mucosal barrier with particulates (bacterial, virus, artificial particulates) and macromolecules. The topic has been reviewed by several authors (see [29–31]).

The essential, protective role of mucus is perhaps most evident in the physiology of the lung, which is continuously exposed to airborne pathogens, toxins, and contaminants. Many of these foreign particles become trapped in the sticky gel of mucus lining the lumen of the bronchoalveolar epithelium and are expelled from the lungs via coughing or cilial motion. In the gastrointestinal tract, the thickness of mucus ranging from 700 μm in the stomach and large intestine to a diameter between 150 and 300 μm in the small intestine [32]. The secreted mucus forms two layers,

a thinner inner layer that is sterile and difficult to dislodge and an outer layer that is not sterile and is more easily removed. Normally, anaerobic commensal microorganisms live in outer mucus layer, leaving the inner mucus layer effectively sterile.

The major components of these barriers are mucin glycoproteins that are produced by mucus cells (goblet cells). Secreted mucin were described by McGuckinin as "a secreted glycoprotein with a central domain containing a dense array of *O*-linked oligosaccharides and amino- and carboxy-terminal cystein-rich domains that oligomerize the mucins into a large macromolecular complex, giving mucus its viscous properties." However, mucus is also formed by other molecules involved in host defense against infection like antimicrobial molecules (cationic and amphipathic peptides or lectins) produced by Paneth cells, or secretory antibodies, IgA and IgG, which are produced by B cells in lamina propria of the intestine and are secreted into the mucus by epithelial cells. All mucus components were exhaustively described by McGuckinin (see [29]).

Evidently, pathogens have evolved many ways of evading the mucosal barrier. Among these mechanisms, some allow efficient penetration of the mucus (presence of flagella), production of enzymes that degrade the mucus, modulation of pathways that allow evasion of the barrier (inflammatory and apoptotic), and disruption of the cells that produce the barrier. Finally, a large number of enteric pathogens have evolved strategies to infect the host via the normal physiological sampling of bacteria and particulates that are carried out by M cells that reside in the dome ephithelium. Goblet cells are not present at dome epithelium, so is not covered by thick mucus layer, leaving holes in the mucus barrier. This anatomophysiological particularity has been appointed as an opportunity to the development of mucosal vaccines.

3.4.3 Immunological Tolerance

Epithelial cells are dynamic participants in the mucosal defense. They have been described as working as sensors detecting danger signals like microbial components through pattern recognition receptors such as Toll-like receptors (TLRs) [14]. The epithelial cells respond to the danger signals by producing cytokine and chemokine signals to underlying mucosal cells, such as dendritic cells (DCs) and macrophages, to trigger innate, nonspecific defenses and promote adaptive immune responses [14, 33].

In the intestine, the environment is extraordinarily rich in food antigens and microorganisms that constitute the normal flora. For this reason, there are mechanisms that reduce and modulate the cytokine and chemokine signals to avoid undesirable responses (reviewed in [34–36]) such as mucosal inflammation.

The mucosal surfaces are in a permanent state of alert, but they "adapt" to the presence of foreign microorganisms. As a consequence, vaccines that produce a strong immune response if injected in sterile tissues such as muscle could be ignored when administered through mucosal surfaces [14]. This state of unresponsiveness or so-called immunological tolerance is dependent on the route of administration of the vaccine and has been appointed as one of the biggest challenges for mucosal

vaccine development. Therefore, intended mucosal vaccination strategies should overcome mucosal tolerance mechanisms, and will require a more detailed understanding of the underlying mechanisms behind the phenomenon. Although the phenomenon of oral tolerance has been known for almost a century, the mechanistic basis is still not fully understood. For instance, the molecular mechanism by which the innate immune system distinguishes commensal from pathogenic bacteria is a topic of great interest which is so far not fully understood. Answers to this and others questions will provide vital information for the development of effective oral vaccines. Some review articles about the state of the art of this knowledge have been published recently [13, 21, 22, 37].

Increasing evidence has shown that the induction of mucosal tolerance is related to the pathway for antigen internalization. One important pathway for tolerance might involve passing through intestinal epithelial cells, escaping capture by lamina-propria phagocytes and transport through blood capillaries to the liver [21]. Another important pathway for the entrance of the antigens from the lumen is via dendritic cells, which can intercalate between epithelial cells and sample antigens directly from the intestinal lumen [26]. It was recently demonstrated that the expansion of dendritic-cell populations mediates the enhancement of oral tolerance [38]. Moreover, these unprocessed antigens are carried through the lymphatics to the mesenteric lymph nodes, which have been implicated in oral tolerance [21, 39]. On the contrary, as demonstrated in more recent studies, Peyer's patches appear not to have an important role in the induction of tolerance [40–42], while the uptake of antigens via Peyer's patches is essential for the induction of an immune response and determines the profile of the induced immune response when using particles as oral antigen carriers [43].

Another important observation is the induction of immunological tolerance that can be induced following the administration of a single high dose of the antigen or a repeated exposure to lower doses. These two forms so-called high- and low-dose tolerance are mediated by distinct mechanisms described recently [21]. It is thought that T-cells are the major cell type involved in the induction of mucosal tolerance. It is generally agreed that the status of oral tolerance can be explained by clonal anergy, clonal deletion of T-cells or by active suppression by regulatory T-cells through the secretion of inhibitory cytokines. The most controversial issue is how and where the antigen-specific T-cells in the MLNs first encounter antigen, and Mowat [13] has reviewed several studies addressing this question. According to the same author, however, it seems more probable that presentation of the antigen to naïve T-cells occurs in the MLNs themselves due to unprocessed antigen brought there by APCs that traffic to the MLNs after being loaded with antigen in the mucosa or Peyer's patches [13].

3.5 Mucosal Adjuvants

To circumvent or minimize these barriers, vaccine formulations and delivery strategies have to be carefully designed in order to efficiently stimulate the innate and adaptive immune response appropriate for the target pathogen [14, 44]. Following this idea,

delivery strategies are likely to be most promising when they mimic pathogens. Therefore, particulate delivery systems that adhere to mucosal surfaces or even better that would be able to selectively target M-cells are likely to be the most effective [14]. Moreover, to be distinguished from commensal microorganisms, the vaccine formulations should also carry substances that activate innate signaling pathways in the epithelial cells and/or in the underlying antigen-presenting cells. These substances which are included in vaccine formulations with the aim of enhancing its immunogenicity are termed adjuvants (*adjuvare*; latin, to help). Presently, there is no optimal adjuvant classification. Although the complete working mechanism of many adjuvants is not entirely known at the moment, classification based on their mode of action has been suggested [45, 46]. Increasing evidence has demonstrated that most non-particulate mucosal adjuvants act by binding to specific receptors, and this adjuvant-class is frequently named immunopotentiators. Particulate adjuvants mainly function to concentrate vaccine components and to target vaccines towards APCs or carry out a depot action.

3.5.1 *Micro- and Nanoparticles as Polymeric Vaccine Delivery Systems*

The category of particulate carriers includes different particles which have been widely reviewed in the recent scientific literature, including microemulsions (such as MF59) [46, 47], iscoms [48, 49], liposomes [48], virosomes [50], virus-like particles, and polymeric microparticles [46, 51–55]. These particles have a common feature, which is that their size should be similar to the size of a pathogen in order to be taken up by APCs [56, 57] and subsequently deliver the associated antigen into these cells. Therefore, the main role of the delivery systems is to concentrate the antigen in the lymphoid tissues responsible for the induction of the immune response. However, the potency of these delivery systems can be significantly improved by the association of an immunopotentiator. This aspect is of particular importance for recombinant vaccines and other weak antigens. Regarding oral and nasal vaccination, the entrapment of vaccine antigens in delivery systems has two main purposes. The first goal is to protect the antigen against degradation on mucosal surfaces, and the other is the enhancement of their uptake in MALT. The most successful work in achieving these two goals has been done with nano- and microparticles. The interaction between particulates and the GALT has been a subject of several reviews [58–61] since a deep understanding of this interaction would be key in the design of successful nanoparticles. The uptake of inert particles has been shown to take place transcellularly through normal enterocytes and specialized M-cells or to a lesser extent across paracellular pathways through the tight junctions between cells [59]. Although transport by the paracellular route has been shown, for example, with polyalkylcyanoacrylate nanocapsules in the jejunal mucosa of the rat [62], the probability of its incidence does not seem to be high since the opening diameter of the gap junctions between the cells is between 7 and 20 nm in diameter [59].

Regarding the transcellular transport, its occurrence via M-cells appears to be a very natural mechanism since M-cells are specialized for endocytosis and subsequently transport the particulates to the adjacent lymphoid tissue (Peyer's patches in the gut). Therefore, after the particle binds to the M-cell apical membranes, the particulates are rapidly internalized and offered to the continuous lymphoid tissue. Depending on their size, the particles can be retained within the lymphoid tissue (>3 μm) [58], or they can be internalized by phagocytic cells and subsequently transported to another lymphoid tissue through the lymphatic vessels that innervate the PP dome area. There is a broad consensus that M-cells, associated with Peyer's patches are the main target for vaccination purposes. However, several questions have arisen regarding this issue. One issue is related to the number of Peyer's patches in the gut and therefore the total area covered with M-cells. Mice and rats have between 6 and 10 discrete Peyer's patches, while a human being has many hundreds [63]. In this respect, the differences between mice and men mean that one must take extreme caution when extrapolating from animal models to humans. On the other hand, these uptake studies have been performed in a small target area in the animal models. Another question is related to the factors that may influence the particle uptake across the gastrointestinal tract epithelium. Some examples reviewed in references [58, 64] are the particle size, ideally it should be smaller than 10 μm for being take up by M-cells of Peyer's patches in intestine and hydrophobicity, increasing the surface hydrophobicity of particles, permeability through mucin also increase whilst decreasing translocation across the cell interior, which has a more hydrophilic environment. Particle surface charge seems to be also an important factor; theoretically, positively charged particles are better positioned to interact with the negatively charged mucin. Additionally, other factors that may influence uptake studies are particle dose, administration vehicle, animal species and age, feeding state of the animals, use of penetration enhancers and use of targeting agents.

3.5.2 *Immunopotentiators*

Nonmicrobial particles, macromolecules, and protein-subunit antigens generally induce weak or undetectable adaptive immune responses when applied mucosally. The encapsulation of the antigen in a particulate delivery system can direct the antigen to the inductive site, ideally to the Peyer's patches, but may not be sufficient to evoke an appropriate immune response, because it may not be recognized as a harmful particulate. To be distinguished from harmless substances and nutrients, mucosal vaccines should raise alarms in the mucosa by including substances in the formulations that activate innate signaling pathways [14].

The best-known mucosal immunopotentiators are the secreted enterotoxins of *V. cholerae* and *E. coli*, cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT). Both CT and LT are exceptionally potent oral-mucosal immunogens (their mechanisms are reviewed in [65]). However, this kind of adjuvants has been shown to be toxic

for humans. Therefore, several genetically modified forms have been engineered to reduce or eliminate the toxicity associated with the enzymatic A subunits of these toxins [66, 67]. In spite of this, some concerns have recently been raised about the use of CT- or LT-derived adjuvants for use in intranasal vaccines. This was based on reports from studies in mice that were intranasally administered CT and LT. These compounds could be localized in the olfactory bulb of the brain, apparently as a result of retrograde transport via the olfactory nerve [68].

Furthermore, many live attenuated mucosal vaccine vectors, including poliovirus, adenovirus, and enteric bacteria are currently under development and have been extensively reviewed [69, 70]. A practical advantage of these live antigen delivery systems is that it avoids the effort and cost associated with antigen purification. Although the superiority of these live attenuated pathogens as mucosal vaccine vectors is due in part to their ability to target the antigen to the appropriate tissue, enhance its uptake to yield a more robust immune response and activate multiple innate immune responses, some safety (virulence reversion) and ethic issues associated with genetic manipulation will delay their use in humans. Therefore, the same safety concerns observed for the live attenuated vaccines already in the market for more than forty years.

Meanwhile, with the recent progress in this area, a number of immunopotentiators have become available for inclusion in vaccines, which have been extensively reviewed elsewhere [46, 71, 72]. Moreover, in more recent years, new information about the functions of immunomodulatory cytokines and the discovery of TLRs has provided promising new alternatives. It has also been demonstrated that the vertebrate innate immune system uses pattern recognition receptors, including TLRs, specifically to detect pathogen-associated molecular patterns (PAMPs) present in infectious agents [73]. To date, at least ten different human TLRs have been identified, as well as a number of naturally occurring TLR ligands. For example, various TLR ligands including CpG-containing oligonucleotides [73], flagellin [74], and bacterial porins [75] have shown adjuvant activity when administered mucosally together with antigens. Synthetic TLR ligands have also been identified, including imidazoquinoline compounds such as imiquimod and resiquimod (R-848), which activate human TLR7 and TLR8 [73] as well.

3.6 Final Remarks

Most pathogens gain access to their hosts through mucosal surfaces. The induction of helpful specific antigen mucosal antibodies is feasible only when the antigen is administered by one of the mucosal routes. On the other hand, a number of obstacles must be overcome in order to efficiently stimulate innate immune responses and evoke adaptive immune responses without disturbing mucosal homeostasis or inducing tolerance. Tolerance mechanism is maybe the most important obstacle. Pathogenic bacteria and virus normally surpass this barrier and therefore theoretically attenuated virus or bacteria are the ideal antigen producers and vectors. Inspired by these vectors, polymeric carriers can be designed in order to have similar sizes as the pathogens, and may be loaded with antigens and immunopotentiators

molecules that will activate innate immune response. Therefore, the investigation of novel nontoxic adjuvants, like delivery systems and immunopotentiators, which should be efficacious on mucosal surfaces is urgently required and is as important as the investigation of new antigens for the development of new vaccines.

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Chapter 4
Oral Vaccination: Attenuated and Gene-Based

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4.1 Introduction

The ability to deliver vaccines by a pill, capsule, chewable candy, or even as a liquid slurry represents a delivery improvement over injected vaccines. Besides the pain of watching our young children return from the pediatrician with multiple band-aids on their legs and tears in the eyes, vaccines that can be administered in the absence of needles have several advantages. Distribution and manufacturing are greatly simplified. A pill can be handed out by anyone, not necessarily by qualified medical support. No sterile filling of syringes or vials is necessary because the stomach and intestinal track handle non-sterile food all the time. Unwanted needle sticks and sharps disposal are avoided. From a performance improvement standpoint, delivering a vaccine mucosally could improve the immune responses mucosally since 90% of pathogens invade by this route and parenteral delivery is not particularly adept at inducing immunity at a mucosal surface. Several approved oral vaccines have been developed, and several oral platform approaches are under investigation that might expand the available pool of vaccines. This chapter reviews the history of oral vaccines, both approved vaccines and those in early stages of development.

4.2 Attenuated Pathogens Given Orally to Prevent Infection

This section describes the use of classical oral vaccines and the infections they prevent. All of these vaccines (Polio, Rotavirus, Typhoid, and Cholera) rely on attenuating pathogens that use the oral route as their natural route of infection.

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