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Expert Review

Mucosal Vaccines: Recent Progress in Understanding the Natural Barriers

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Abstract. It has long been known that protection against pathogens invading the organism via mucosal surfaces correlates better with the presence of specific antibodies in local secretions than with serum antibodies. The most effective way to induce mucosal immunity is to administer antigens directly to the mucosal surface. The development of vaccines for mucosal application requires antigen delivery systems and immunopotentiators that efficiently facilitate the presentation of the antigen to the mucosal immune system. This review provides an overview of the events within mucosal tissues that lead to protective mucosal immune responses. The understanding of those biological mechanisms, together with knowledge of the technology of vaccines and adjuvants, provides guidance on important technical aspects of mucosal vaccine design. Not being exhaustive, this review also provides information related to modern adjuvants, including polymeric delivery systems and immunopotentiators.

KEY WORDS: adjuvants; immunoglobulin A; mucosal immune system; mucosal vaccines.

INTRODUCTION: A BRIEF HISTORY OF VACCINES AND ADJUVANTS

The scientific era of vaccinology started in the early eighteenth century, with the introduction in Europe of an ancestral Chinese practise of preventing severe natural smallpox by inoculating pus from smallpox patients. This procedure was introduced to England for the first time by a farmer named Benjamin Justy who inoculated his family with cowpox pus to prevent smallpox, and the first clinical investigations were eventually conducted in 1796 by the English practitioner Edward Jenner (1). During the fifteenth century in China, healthy people acquired immunity to smallpox by sniffing powdered smallpox pustules, by inserting them into small cuts in the skin (a technique called variolation) (2), or finally by the oral administration of fleas from cows with cowpox. These are the first reports of a mucosal vaccination practice (3).

A great expansion of biomedical sciences and vaccinology occurred in the nineteenth century with the enormous contribution by Louis Pasteur of the first attenuated vaccine (from Latin *vacca*: "cow") and with the achievements of Robert Koch; Emil von Behring, the first recipient of the Medicine Nobel Prize; and Paul Ehrlich. Between World Wars I and II, many studies were carried out which led to the description of most kinds of humoral immunologic phenomena (1). Although that period also had many financial restrictions, since resources were principally used for military purposes, this early period led to the appearance of the first vaccines against typhoid fever, shigellosis, tuberculosis, plague, diphtheria and tetanus.

The modern era of vaccine science began in about 1950 with the bacterial capsular polysaccharide vaccines such as *pneumococcus*, *meningococcus* and *Haemophilus influenzae*. Moreover, the Sabin oral polio vaccine in the early 1960s had an important role in the programme for the global eradication of polio and brought mucosal immunization to prominence (3). The appearance of viral vaccines such as the inactivated poliovaccines and live vaccines for preventing pediatric diseases, measles, mumps, rubella and varicella vaccine also played important roles in the history of vaccines. More recently, the discovery of vaccines against hepatitis in the early 1960s was initiated with the purpose of discovering the etiological agent causing hepatitis A and B. Blumberg and colleagues in 1965 discovered the surface antigen of the hepatitis B virus present in the blood of human carriers of the

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ABBREVIATIONS: APCs, antigen presenting cells; BALT, bronchus-associated lymphoid tissue; CCL25, chemokine ligand 25; CCL28, chemokine ligand 28; CCR9, chemokine receptor 9; CCR10, chemokine receptor 10; CMIS, common mucosal Immune system; CTL, cytotoxic T lymphocyte; DCs, dendritic cells; FAE, follicle-associated epithelium; GALT, gut-associated lymphoid tissue; HBV, Hepatitis B virus; IELs, intraepithelial lymphocytes; MADCAM1, mucosal adressin cell-adhesion molecule1; MALT, mucosa-associated lymphoid tissues; M cells, microfold epithelial cells; MHC, major histocompatibility complex; MLN, mesenteric lymphoid nodes; PAMPs, pathogen-associated molecular patterns; PLG, polylactide-co-glycolide; sIgA, secretory immunoglobulin A; TECK, thymus-expressed chemokine; TGTß, transforming grown factor; TH2, T helper 2 cells; TLRs, Toll-like receptors; VCAM1, vascular cell adhesion molecule 1.

infection (1). This discovery opened the door to a hepatitis B vaccine, which has been considered among the most remarkable scientific achievements of the 20th century (4). According to Hilleman (1), hepatitis vaccines represent the world's first subunit vaccine, the world's first licensed vaccine against human cancer and the world's first recombinantly expressed vaccine.

Detailed reviews focusing on the history of the hepatitis B vaccine can be found in the scientific literature (1,4–7). In brief, the plasma-derived hepatitis B vaccine was licensed in 1981, 16 years after Blumberg's discovery. The main reason for this delay was the inability to propagate HBV in tissue culture systems. The pioneering work of Krugman and colleagues (8) made feasible the production of a vaccine containing hepatitis B surface antigen obtained by purification of the serum of asymptomatic chronic HBV carriers and characterized elsewhere (9–12), which was decisive for the development of the vaccine.

The hepatitis plasma-derived vaccines successfully immunized several million individuals world-wide over almost a decade. However, because these vaccines had a poor acceptance rate due to concerns regarding the safety of the plasma-derived products and because the supplies of acceptable human carrier plasma were inadequate to meet market needs, recombinant DNA techniques were investigated as an alternative production method. Therefore, a yeast-derived hepatitis B vaccine based on recombinant DNA technology was licensed in 1986, and its properties have been reviewed elsewhere (13–15).

According to Hilleman (1), contemporary vaccinology research is very complex, at least for viral vaccines, and is largely dedicated to the subunit vaccine approach. Moreover, subunit vaccines are built on the same base and may be considered to be an extension of recombinant subunit hepatitis B technology, one example being the investigation of a vaccine against AIDS. The discovery of new vaccines to control more than 20 diseases, especially malaria, tuberculosis, hepatitis C and AIDS, relies on the identification of appropriate antigens and epitopes, and progress towards this goal will benefit from the expansion of knowledge in the fields of immunology and molecular biology.

The major questions for current vaccine research seem to ask "what to present to the immune system" and "how to present it" (1). The answer to the last question will depend on the parallel development of new, safe and efficient adjuvants.

MUCOSAL VACCINATION

Mucosal vaccination has been the common generic name attributed to the oral, intranasal, pulmonary, rectal and vaginal routes of vaccine administration. However, the mucous membranes do not only cover the aerodigestive and urogenital tracts, but also the eye conjuntiva, the inner ear and the ducts of all exocrine glands, which have been less explored as routes of vaccine administration.

Mucosal surfaces, with a combined surface area of about 400 m^2 (2), 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 (16). 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 (2).

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 (17). However, even with the many attractive features of mucosal vaccination described below (Table I), it has often proven difficult (Table II) in practice to stimulate strong sIgA immune responses and protection by mucosal antigen administration (16). As a consequence, no more than half a dozen mucosal vaccines are currently approved for human use (Table III), and no subunit vaccines are listed among those approved.

Table I. Additional Advantages of Oral and Nasal Vaccination

	Oral	Nasal
Does not require injection and is therefore less painful	X	X
Has a high patient compliance among infants and adults	X (3)	
Does not require trained medical personnel for delivery and is thus more appropriate for mass vaccination programmes, especially in under-developed countries, and would also be a benefit in pandemic and bioterrorism situations	X	X
Has a natural route of administration	X	
Has higher stability resulting from solid oral formulations of vaccines	X	
Can induce mucosal antibodies (sIgA)	X	X (21)
Uses the most effective route to elicit optimal protective immunity in both mucosal and systemic immune compartments		X (21)
Can avoid the degradation of the vaccine antigen caused by digestive enzymes requiring a smaller antigen dose than oral immunization		X (3,21)
Can generate cross-protective immunity in the gut through the common mucosal immune system		X (21)

Table II. Challenges in Mucosal Vaccine Design

	Oral	Nasal
May lead to a possible deposition of antigen in the central nervous system through the olfactory bulbs and olfactory nerves (this feature requires further investigation, however should not be excluded)		X (21)
Has a low ability for the antigens to be taken up by the mucosal immune system	X (17)	X (17)
May allow for gastrointestinal deactivation of the vaccines requiring high doses of the vaccine	X (3)	
It associated with a high variability of the response and mixed clinical data	X (3)	
Features high clearance in nasal mucosa, which is a cause of low absorption of biomacromolecules		X
Has significant enzymatic activity in mucosal tissues	x	X (3)
Has limited applicability in patients with upper respiratory-tract infections		X (3)
May be associated with occurrence of induction of immunological tolerance (discussed below)	X	X

UNDERSTANDING THE ANATOMOPHYSIOLOGY OF THE MUCOSAL IMMUNE SYSTEM

Mucosal-associated lymphoid tissue (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 mucosa-associated lymphoid tissues (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 (18,19). However, the extent of the immune response at the effector sites depends on where the induction occurred (20). Holmgrenn and Czerkinsky (16) 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." Kiyono (21) recently referred to important evidence that may explain, at least in part, the dependence of the mucosal site where the IgA is generated on the route of antigen administration. Nasal immunization induces the expression of high levels of chemokine receptor 10 (CCR10) and $\alpha_4\beta_1$-integrin by IgA-committed B cells, allowing them to efficiently traffic to the respiratory and genito-urinary tracts, which express the corresponding ligands, chemokine ligand 28 (CCL28) and vascular cell adhesion molecule 1 (VCAM1) (22). In contrast, orally induced IgA-committed B cells express CCR9 and CCR10 as well as $\alpha_4\beta_7$ and $\alpha_4\beta_1$-integrins, so the cells migrate to sites such as the small intestine, which express CCL25 and/or CCL28 together with mucosal addressin cell-adhesion molecule -1 (MADCAM1) and/or vascular cell adhesion molecule-1 (VCAM1) (22). Therefore, despite the fact that NALT and Peyer's patches are apparently colonized by similar immune cells, subtle differences like the example referred to above indicate that these two lymphoid structures may have somewhat different biological functions which are most probably related to their anatomically and environmentally distinct locations (21).

This work will focus on the GALT and NALT. In particular, we will focus on Peyer's patches and NALT as the inductive sites, and the effector sites will be briefly described as well. Effector sites include the lamina propria of the intestinal and respiratory tracts responsible for the generation of antigen-specific T helper 2 (Th2)-cell-dependent IgA responses and Th1-cell and cytotoxic T lymphocyte (CTL)-dependent immune responses, which function as the first line of defence at mucosal surfaces.

Gut-Associated Lymphoid Tissue (GALT)

The gut-associated lymphoid tissue described elsewhere (2) (Fig. 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 (2)).

Table III. Licensed Mucosal Vaccines (Adapted from Ref (16))

Infection	Vaccine	Route
Polio	Live attenuated vaccine (OPV)	Oral
Cholera	Cholera toxin B subunit+inactivated *V. cholerae* O1; whole cells	Oral
Cholera	CVD 103.HgR live attenuated *V. cholerae* 01 strain	Oral
Typhoid	Ty21a live attenuated vaccine	Oral
Rotavirus	Live attenuated monovalent human rotavirus strain	Oral
Influenza	Live attenuated cold-adapted influenza virus reassortant strains	Nasal

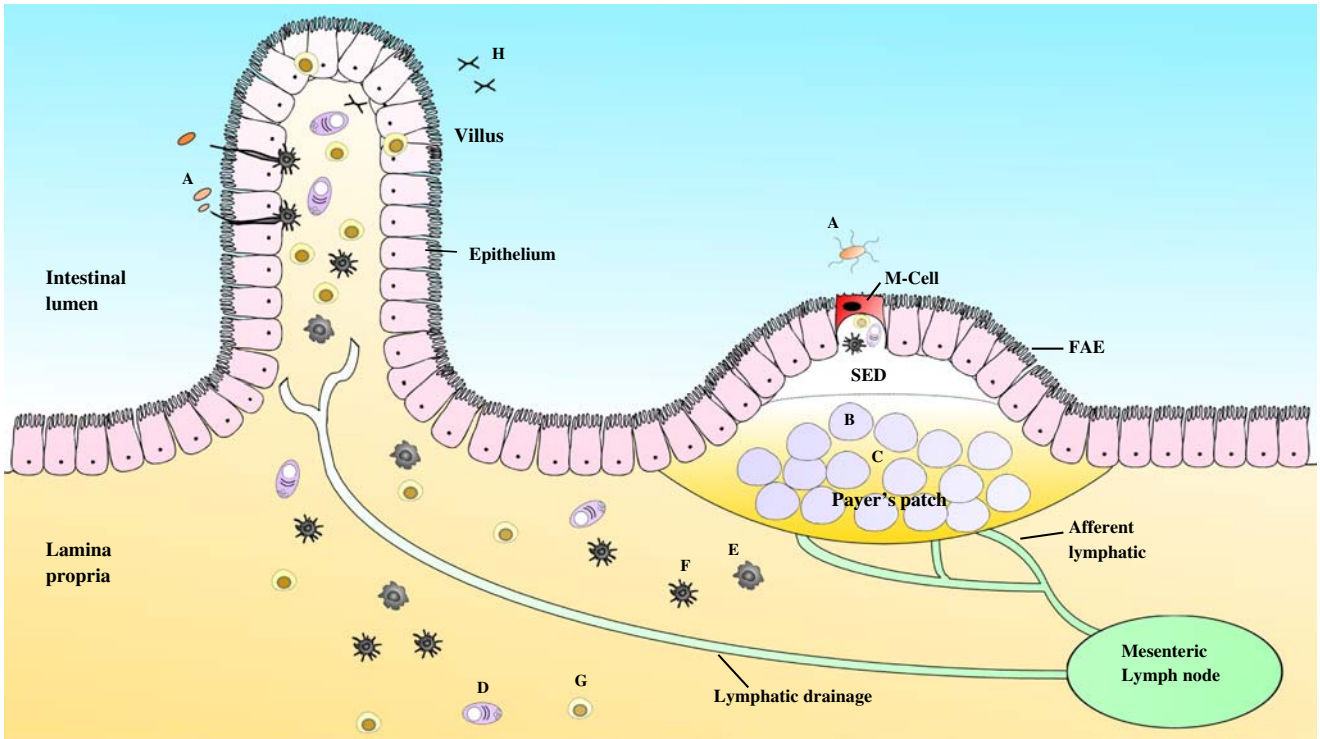


Fig. 1. Schematic representation of the gut-associated lymphoid tissue (GALT). Under the epithelial layer is the lamina propria, which contains a large number of B cells, plasma cells, dendritic cells and macrophages. In the submucosal layer underneath the lamina propria are located the Peyer's patches, which contain lymphoid follicles. Between the follicle-associated epithelium (FAE) and the organized lymphoid follicle aggregates, there is a more diffuse area known as the subepithelial dome (SED). FAE is a small region characterized by the presence of specialized flattened epithelial cells called M cells. A — Antigen; B — Lymphoid follicle; C — Parafollicular T— lymphocyte zone; D — Plasma Cell; E — Macrophage; F — Dendritic Cell; G — Lymphoid cell; H — sIgA.

Peyer's patches, located in the submucosal layer underneath the lamina propria, contain 30–40 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 (23).

The function and structural characteristics of microfold epithelial cells (M cells) have been described in several recent reviews (2,23). 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, if not the only, route for complex antigens and pathogen invasion, for example, several invasive *Salmonella* species, *Vibrio cholerae, Yersinia* species, *Escherichia coli* and the polio virus (23).

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 humans. In mice and humans, 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 (23). The epithelium of the gut intestine provides an effective barrier to the entrance of most pathogens and particulates due to strong connections between epithelial cells called tight junctions. In contrast, the M cells can be exploited by microorganisms as the port of entrance for two reasons: the organisms can adhere with less difficulty to the apical cell membrane, and after that, these agents need only be transported a short distance before reaching the M cell pocket, where by interaction with lymphocytes, the antigens or the

particulates gain rapid access to the organized MALT inductive site.

Nasopharynx-Associated Lymphoid Tissue (NALT)

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) (24). In the rat, lymphoid aggregates are situated at the nasal entrance to the pharyngeal duct (25). Detailed reviews of NALT and nasal vaccination can be found elsewhere (25–27). NALT is a well-organised structure consisting of B- and T-cell-enriched areas, which are covered by an epithelial layer containing microfold M cells, the so-called follicle-associated epithelium (FAE). The function of these antigen-sampling M cells seems to be similar to those found on the FAE of Peyer's patches (21). Although NALT and Peyer's patches share certain similarities, the two differ markedly in morphology, lymphoid migration patterns and the binding properties of the [high] endothelial venules (24). Additionally, intraepithelial lymphocytes and antigen-presenting cells, including dendritic cells (DCs) and macrophages, can also be found in NALT (28). Therefore, according to Kiyono (21), 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.

NALT- AND PEYER'S PATCH-INITIATED IMMUNE RESPONSES

Several evidences converge on the insight that the organized 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 implicates active lymphocyte proliferative activity, local production of cytokines and continuous cellular trafficking (29).

In stratified and pseudostratified epithelia (which lack tight junctions), antigen-processing dendritic cells move into the epithelium, internalise antigens from the lumen and migrate back to local or distant organized tissues. In the intestinal and airway epithelia, whose mucosal epithelial cells are sealed by tight junctions, antigen 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 (29,30). Simultaneously, it is believed that the intact antigens are processed by professional 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 (17). Phenotypically immature DCs are subsequently moved to the T-cell areas, where they upregulate the expression of maturation markers and MHC molecules (17).

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 (30). 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 (31). 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 micro-architectural 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 (32). 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 (21,32).

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 (17,22,33). For example, the exit of the lymphocytes into the mucosa occurs because lymphocytes that are primed by antigen in the GALT lose expression of L-selectin and selectively upregulate the expression of $\alpha_1\beta_7$ integrin. This guides the emigration of lymphocytes from the bloodstream by interacting with mucosal addressin cell-adhesion molecule 1 (MADCAM1) (30). Another example refers to the expression of the chemokine receptor-9 (CCR9), induced by gut-derived T cells that respond to the chemokine ligand-25 (CCL25), also known as TECK (thymus-expressed chemokine), which is expressed selectively by small bowel epithelial cells. On the contrary, T cells primed in peripheral lymphoid organs cannot migrate to mucosal surfaces because they do not express the same molecules.

IgA-secreting B cells that are activated in MALT express CCR10, the receptor for CCL28. Therefore, CCR10+IgA+B cells can be attracted by all tissues containing CCL28-secretor epithelial cells, which include the small and large intestines, salivary glands, tonsils, respiratory tract and lactating mammary glands (22). This mechanism explains why mucosal immunization at one site can result in the secretion of specific IgA antibodies in other mucosal or glandular tissues. On the other hand, there are also some receptor-mediated recognition systems that have a more selective function. For example, IgA+B cells that are generated in the intestinal inductive sites express the homing receptor $\alpha_4\beta_7$-integrin that

interacts strongly with MADCAM1, an addressin that is expressed by venules in the small and large intestines and in lactating mammary glands, but not in other mucosal tissues (22). The reason that IgA+B cells which are activated in the peripheral lymph nodes following systemic immunization do not migrate to mucosal sites seems to be related to their inability to express the CCR10, $\alpha_4\beta_7$-integrin and other mucosal "homing receptors."

Mucosal antigen delivery can either up-regulate or down-regulate systemic immune responses. Therefore, the understanding of both mechanisms will provide better guidance on the technical aspects for mucosal vaccine design.

Production of Immunoglobulin A (IgA)

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 (2). 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 (17). The IgA of external secretions, called secretory IgA (sIgA) (Fig. 2), consists of a dimer or tetramer, a j-chain polypeptide and a polypeptide chain called the secretory component (2,34). This sIgA is resistant to degradation in the protease-rich external environment of mucosal surfaces. The resistance is due to its dimerization and high degree of glycosylation during its synthesis in mucosal plasma cells, and by its association with a glycosylated fragment (the secretory component) (17).

The secretory immunoglobulin A has several functions in mucosal defence described elsewhere (16,17,21). 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 (17,35). Additionally, specific sIgA might block or sterically hinder the microbial surface molecules that mediate epithelial attachment (36).

Immunological Tolerance

Epithelial cells are active participants in the mucosal defence. They have been described as functioning as sensors detecting dangerous signals like microbial components through pattern recognition receptors such as Toll-like receptors (TLRs) (17). The epithelial cells respond to the dangerous signals by producing cytokine and chemokine signals to underlying mucosal cells, such as dendritic cells (DCs) and macrophages, to trigger innate, non-specific defences and promote adaptive immune responses (17,37).

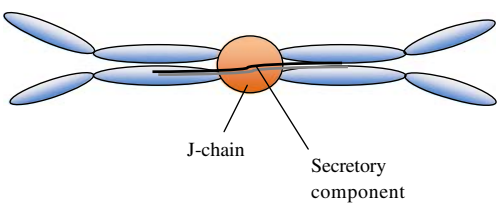


Fig. 2. Secretory IgA (sIgA) — sIgA consists of a dimer or tetramer, j-chain polypeptide and a polypeptide chain called the secretory component.

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 (38–40)) 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 (17). This state of unresponsiveness or so-called immunological tolerance is dependent on the route of administration of the vaccine (see Table IV) and has been appointed as one of the bigger 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 understood. Answers to this and other 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 (30,41); therefore, only a short summary concerning immunological tolerance is presented here.

Increasing evidence has shown that the induction of mucosal tolerance is related to the path 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 (41). 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 (42). It was recently demonstrated that the expansion of dendritic cell populations mediates the enhancement of oral tolerance (43). Moreover, these unprocessed antigens are carried through the lymphatics to the mesenteric lymph nodes, which have been implicated in oral tolerance (41,44). On the contrary, as demonstrated in more recent studies, Peyer's patches appear not to have an important role in the induction of tolerance (45–47), although 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 (48).

Another important approach for the induction of immunological tolerance is the administration of a single high dose of the antigen or repeated exposure to lower doses. These two forms of tolerance, now the so-called high- and low-dose tolerance, are mediated by distinct mechanisms as described recently (41). 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 T regulatory cells through the secretion of

Table IV. Route of Antigen Administration Affects Immunological Response (Adapted from Ref. (41))

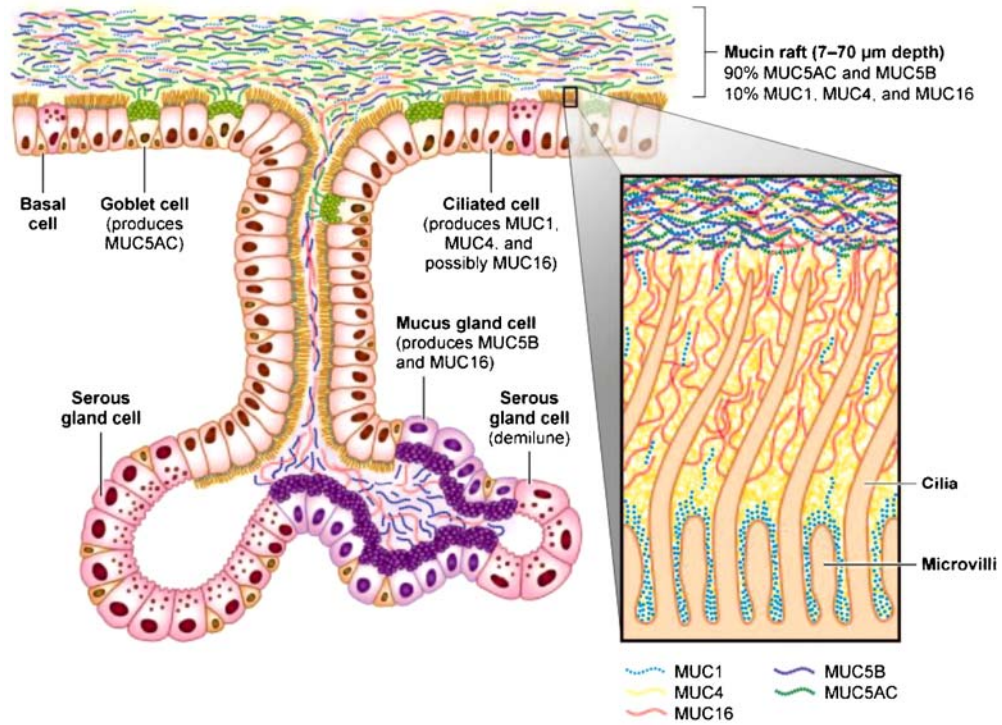
Route of antigen administration	Usual outcome
Subcutaneous	Immunization
Intramuscular	Immunization
Injury	Immunization
Intravenous	Tolerance
Mucosal (oral, nasal and pulmonary)	Tolerance
Portal vein	Tolerance
Anterior chamber of the eye	Tolerance

inhibitory cytokines. The most controversial issue is how and where the antigen-specific T cells in the MLNs first encounter antigen, and Mowat (30) 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 (30).

CHALLENGES IN ORAL AND NASAL VACCINE DESIGN

Vaccines administered mucosally encounter the same host defence barriers as microbial pathogens and other foreign macromolecules: they are diluted in mucosal secretions, detained in mucus gels (excellent represented on Fig. 3), attacked by proteases and nucleases and barred by epithelial barriers (17). 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 generally induce immune tolerance (41).

To circumvent or minimize these difficulties, 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 (17). 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 (17). Moreover, to be distinguished from commensal microorganisms, the vaccine



Hattrup CL, Gendler SJ. 2008.
Annu. Rev. Physiol. 70:431–57

Fig. 3. This figure and legend were previously published by Hattrup CL, Gendler SJ. on "Structure and function of the cell surface (tethered) mucins" Annu Rev Physiol. 2008;70:431–57. Published in this journal with the permission of authors and Journal. "Mucins on the respiratory epithelium. Ciliated cells and goblet cells in surface epithelium and mucus gland are shown in a simplified representation of the airway epithelium. Tethered mucins (MUC1, MUC4, and MUC16) are found both in the normal, cell-associated form as well as a secreted form in the overlying mucin raft along with MUC5AC and MUC5B. The mucin raft is composed mainly (90%) of MUC5AC (from goblet cells) and MUC5B (from mucus glands). The tethered mucins (MUC1, MUC4, and MUC16) make up approximately 10% of the mucus raft and may result from shedding and alternative splicing. The figure is adapted from Sheehan *et al.* (10) and based on personal communications with Drs. John Sheehan and David Thornton."

formulations should also carry substances that activate innate signalling pathways in the epithelial cells and/or in the underlying antigen-presenting cells. These substances that are included in vaccine formulations with the aim of enhancing its immunogenicity are termed adjuvants (from Latin *adjuvare*: "to help") (see Table V). 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 (49,50). 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 antigen presenting cells (APCs) or carry out a depot action. Therefore, this section will briefly review existing mucosal adjuvants, mainly dealing with those which have been used in the present experimental work of this thesis, so, chitosan-based particulate delivery systems and CpG oligodeoxynucleotides are described in the following chapters.

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) (50,61), iscoms (62,63), liposomes (62), virosomes (64), virus-like particles and polymeric microparticles (50,65–69). 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 (70,71) 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 immune response induction. However, the potency of these delivery systems can be significantly improved by the association of an immunopotentiator (see 5.2) (72). This aspect is of particular importance for recombinant vaccines and other weak antigens.

Therefore, there is a huge amount of information about the interaction of immune cells with different compounds (immunopotentiators) and particulate delivery systems, which allow for vast combination possibilities to be used in adjuvant formulations. According to O'Hagan (50), we are entering an exciting and dynamic time in vaccine research in which the principles leading to the successful induction of potent and protective immune responses are becoming better understood. This explosion of knowledge is not only for the traditional parenteral routes of vaccine administration but also for mucosal vaccination. 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 (73–76), and 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 (74). Although transport by the paracellular route has been shown, for example, with polyalkylcyanoacrylate nanocapsules in the jejunal mucosa of the rat (77), 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 nm and 20 nm in diameter (74).

Regarding the transcellular transport, its occurrence via M cells appears to be a very natural mechanism since M cells are specialised 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 (>3μm) (73), 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 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 (78). In this respect, the differences between mice and humans 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 (73,79) are the particle size—ideally it should be smaller than 10μm 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 increases whilst decreasing translocation across the cell interior, which has a more hydrophilic environment. Particle surface charge seems to also be an important factor; theoretically, positively charged particles are better positioned to interact with the negatively charged mucin. Additionally, others 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.

Table V. Classification of Vaccine Adjuvants (Adapted from (50))

Antigen delivery systems	Immunopotentiators
Alum (51,52)	MPL and synthetic derivatives
Calcium phosphate (53)	MDP and derivatives
Tyrosine (54)	CpG oligos
Liposomes (55)	Alternative PAMPS - flagellin
Virosomes (55)	Lipopeptides
Emulsions (56)	Saponins
Microparticles (57)	DsRNA
Iscoms (58)	Small-molecule immunopotentiators
Virus-like particles (59)	Mast cell activators (60)

A number of polymeric delivery systems have been evaluated by mucosal routes; however, most of the work in this area has focused on poly(lactide-co-glycolide) (PLGs) polymers (some examples in (80–86)). These polymers are biodegradable and biocompatible, and there is already a much experience using them as a suture material in humans (79). Moreover, they have appropriate release characteristics for use in single-dose vaccines (87). One of the limitations of this polymer is its insolubility in water, which makes the use of organic solvents necessary in particle preparation. Additionally, during the manufacturing process of the particles, the antigen may also be exposed to high shear stress, aqueous/organic interfaces and elevated temperatures, which have been considered extreme conditions for working with proteins and antigens. More recently, a different approach was adopted with these nanoparticles, as the antigen was adsorbed onto the cationic modified surface of PLG nanoparticles after their preparation (88,89).

Although PLGs have been successfully used for the entrapment of several antigens, the investigation of new polymeric delivery systems produced in a harmless environment has emerged over the last years. Some examples include the preparation of alginate microspheres for the entrapment of rotavirus (90), polyacryl starch for the entrapment of salmonella (91) and chitosan nanoparticles for the entrapment of diphtheria (62).

Immunopotentiators

Non-microbial 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 signalling pathways (17).

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 mechanism are reviewed in (92)). 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 (93,94). 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 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 (95).

Furthermore, many live attenuated mucosal vaccine vectors, including poliovirus, adenovirus and enteric bacteria are currently under development and have been extensively reviewed (96,97). A practical advantage of these live antigen delivery systems is that they avoid 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 ethical issues associated with genetic manipulation will delay their use in humans. The same safety concerns were observed for the live attenuated vaccines already in the market for more than 40 years.

Meanwhile, with the recent progress in this area, a number of immunopotentiators have become available for inclusion in vaccines (see Table VI), which have been extensively reviewed elsewhere (50,72,98). Moreover, in more recent years, new information about the functions of immunomodulatory cytokines and the discovery of Toll-like receptors (TLRs) have 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 (99). To date, at least ten different human TLRs have been identified, as well as a number of naturally occurring TLR ligands (some examples are described in Table VI). For example, various TLR ligands including CpG-containing oligonucleotides (99), flagellin (100) and bacterial porins (101) 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 (99) as well.

As an example of an already well-studied immunopotentiator, the B-class CpG ODN has been frequently used in animal studies due to their strong B cell activation and capacity to induce potent Th1-type immune response. The same B-class CpGs have also been shown to be safe and efficacious vaccine adjuvants in humans (102,103). Although most cell types have the capacity to internalize CpG ODN via endocytosis (104), only those cells that express the TLR9 are activated. In humans, only B cells and plasmacytoid dendritic cells (pDCs) are able to express the TLR9, whereas in mice, TLR9 is also found on myeloid dendritic cells (mDCs), macrophages and monocytes (99). Within minutes after exposure to CpG ODN, these cells take up the CpG ODN into an endosomal compartment where interaction with the TLR9 occurs (105). This leads to the activation of cell signalling pathways comprehensively described by McCluskie

Table VI. Toll-Like Receptors and Naturally Occurring Ligands

Receptor	Ligand	References
TLR2	Lipoproteins and peptidoglycans	(114,115)
TLR3	Double-stranded RNA of viral origin	(116)
TLR4	Lipopolysaccharide (LPS) from gram-negative bacteria and lipotechoic acid from gram-positive bacteria	(117–120)
TLR5	Flagellin, a protein found in bacterial flagella	(121,122)
TLR7/ 8	Single-stranded viral RNA	(123)
TLR 9	Unmethylated CpG motifs found in bacterial DNA	(124–126)

(99). CpG ODN has been shown to be an effective mucosal adjuvant after administration to different mucosal surfaces, such as the respiratory tract (106–108), the genitourinary tract (109) and the gastrointestinal tract (110,111), in combination with different antigens including the hepatitis B antigen (107,112).

The combination of CpG with other adjuvants has been considered to be useful regarding several issues. One issue is CpG ODN, a strong Th1 profile inducer which has been shown to be able to dominate the Th2 bias associated with adjuvants such as alum or Freund's incomplete adjuvant (FIA) (107,113). Another important advantage is the depot effect offered by several adjuvants that may result in an extended release period during which both antigen and CpG ODN are available. Finally and not less important is the fact that the association of CpG ODN with nanoparticles may protect the CpG ODN from degradation on mucosal surfaces, particularly the ODN synthesized with the native phosphodiester (PO) backbone, which rapidly degrades *in vivo*.

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 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 can be loaded with merely interest antigens and immunopotentiators molecules that will activate innate immune response. Therefore, the investigation of novel non-toxic adjuvants, like delivery systems and immunopotentiators, which should be efficacious by the mucosal surfaces, is urgently required and is as important as the investigation of new antigens.

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