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Review Article

Mucosal adjuvants and delivery systems for oral and nasal vaccination

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Abstract

Mucosal vaccination offers the advantages of blocking pathogens at the portal of entry, improving patient compliance, facilitating vaccine delivery and decreasing the risk of unwanted spread of infectious agents via contaminated syringes. During the last several decades, an enormous expansion in the knowledge of mucosal immunity, cellular immunology and molecular biology has led to new perspectives for creating mucosal vaccines with the ability to induce a balanced systemic and secretory immune response. Better understanding of the mechanisms of action has resulted in an explosion of technologies. In addition to classical aluminium salts, components of microbial origin (DNA motifs, lipid A, bacterial toxins), emulsions and particles (immunostimulating complexes, liposomes, PLGA and saponins), as well as synthetic analogues and cytokines, are examples of adjuvants and delivery systems in use or under preclinical or clinical development.

Introduction

Vaccines represent the most cost-effective and successful life-saving medical instrument ever developed. This has become evident by the dramatic reduction in morbidity and mortality caused by several microorganisms (1, 2) following the introduction of vaccination. However, the World Health Organization (WHO) recently expressed growing concern over the large increase in infections as a result of the use of unsterile needles. About 12 billion preventive and curative injections are given each year, and 40 million every day. Diseases most frequently transmitted through unsafe injection practices are hepatitis B, hepatitis C and HIV/AIDS (3).

The mucosal application of vaccines not only provides an enhanced safety profile, but also offers a number of other advantages over conventional parenteral vaccine regimes. One of the most notable benefits of mucosal immunization is that both systemic and mucosal immunity are triggered, which is particularly advantageous in the case of vaccination against diseases caused by mucosal pathogens. The vast majority of infections occur, or start from, mucosal surfaces, leading to the conclusion that protective immunization against these pathogens requires a successful mucosal immune response. Therefore, the mucosal route appears to be the most appropriate means for immunization. However, most clinically relevant vaccine candidates show weak immunogenicity when delivered mucosally and poor transport characteristics across biological barriers. This implies the need for adjuvants to potentiate the protective immune response or to improve presentation and targeting.

The immune response to pathogens includes two systems of recognition. The first line of defense is innate immunity and the second the adaptive response. Innate cells detect the type of pathogen and play a critical role in the production of cytokines, important in directing and balancing the subsequent protective adaptive response.

Generally, effective vaccines must have three key components: 1) an antigen against which an adaptive immune response is generated; 2) an immune potentiator to signal the innate immune system to favor and/or potentiate the antigen-specific response; and 3) a delivery system to target the antigen and the immune potentiator to the right location. Important features in the design of mucosal vaccine formulations include protection of the antigen from enzymatic digestion at the mucosal surface, enhancement of antigen uptake by M-cells or

conventional epithelial cells in the gastrointestinal tract and respiratory mucosae, stimulation of the innate and appropriate adaptive immune responses, and induction of immunological memory. Attempts to address these objectives include the use of appropriate delivery systems and immunostimulatory molecules which are active when administered by mucosal routes.

The mucosal immune system

The mucosal immune system exhibits two distinct responses: local immunity involving T-cell responses and IgA antibody formation on the one hand, and the development of systemic and local tolerance on the other. Tolerance is the default pathway and the normal response to soluble protein antigens encountered at mucosal membranes, *e.g.,* after feeding or inhalation (4). As a consequence, an active immune response after mucosal antigen administration usually requires the use of potent adjuvants (5) able to overcome or to break tolerance or unresponsiveness.

The mucosal surfaces of the gastrointestinal and respiratory tracts represent the main portals of entry for most human pathogens. The mucosa-associated lymphoid tissue (MALT) was first described almost 3 decades ago (6) and has undergone major investigations since then (7-9). Peyer's patches and other organized lymphoid follicles in the gut-associated lymphoid tissue (GALT) and the nasal-associated lymphoid tissue (NALT) play a critical role in the induction of mucosal immune responses or in the development of systemic hyporesponsiveness following oral exposure to an antigen (oral tolerance) (10-12).

Peyer's patches in the gut are equipped with specialized cells called M-cells. The presence of M-cells on epithelial surfaces appears to be a common feature of all inductive mucosal sites. While the precise mechanism of antigen uptake in mucosal tissues has not been fully established, it is well accepted that M-cells are important in luminal uptake, transport, processing and presentation of mucosally introduced antigens. Active transportation of antigens into subepithelial compartments within the MALT is a prerequisite for the induction of local and/or systemic immune responses (8, 13).

Numerous studies carried out in both animals and humans have shown that orally administered antigens stimulate particular cells, specifically T-helper cells and IgA-producing B-cells in the GALT, and especially in the Peyer's patches, which leads to the dissemination of B- and T-cells via efferent lymphatics and their migration to mesenteric lymph nodes, and then into the thoracic duct to reach the bloodstream. These migrating cells enter the mucosal effector tissues such as the bronchi, the lamina propria regions of the intestine, the genitourinary tract and various secretory glands, where terminal differentiation, synthesis and transport for subsequent antigen-specific secretory IgA (sIgA) responses occur (14-16). This induction in MALT and exodus of cells to effector sites is defined as the common mucosal immune system.

The NALT appears to have better developed lymphoid follicles, with marked intraepithelial infiltration by lymphocytes (17). Intranasal administration of antigens exploits the relatively high permeability of the nasal mucosa compared to other mucosal surfaces. Soluble antigens are able to access the nasal epithelial cells relatively easily, and stimulate immunological responses in draining lymph nodes mediated by intraepithelial dendritic cells (18). Particulate antigens are believed to access the NALT primarily via M-cells and are then processed (19).

Balancing the immune response

Depending on the nature of the invading pathogen, cells of the innate immune system, which include macrophages and dendritic cells, are activated and produce IL-4 or IL-12. This is a key process in directing naïve T-cells to differentiate into Th2 and Th1 cells. Most products of bacteria and viruses, including lipopolysaccharide (LPS), bacterial DNA and dsRNA, drive differentiation towards the Th1 functional phenotype (20, 21). Th1 cells secrete Th1-type cytokines, which include IL-2, interferon gamma (IFN-γ) and TNF-β and drive cell-mediated immunity, such as delayed-type hypersensitivity, macrophage activation and inflammatory responses. Furthermore, Th1 cells can provide a helper function for specific IgG subclasses, particularly those involved in opsonization and virus neutralization (22). In the presence of helminthic pathogens and allergens, naïve T-cells are differentiated into Th2 cells. Th2-type cytokines, including IL-4, IL-5, IL-10 and IL-13, mediate humoral immunity, and in the mouse, they support IgG_1 subclass and IgE production. Either Th1 or Th2 cells, or a combination of both, may be important in favoring antigen-specific secretory IgA responses. Th2-type responses have been shown to be important in the terminal differentiation of B-cells (23), whereas the Th1 cytokine IFN-γ has been implicated in the induction of the polymeric immunoglobulin receptor required for transport of secretory IgA (24). Importantly, Th1 and Th2 T-cells are reciprocally regulated by a range of cytokines produced by the T-cells themselves or by cells of the innate immune system.

A better understanding of how antigen-presenting cells (APCs), particularly dendritic cells and macrophages, direct potent and selective T-cell responses and the discovery of a family of distinct receptors collectively called toll-like receptors, or TLRs (25), have allowed the design of adjuvants to enhance vaccine efficacy (20, 26-29). The TLRs recognize structural components of pathogens, also called pathogen-associated molecular patterns (PAMP), and activate cells. Within minutes following recognition of PAMPs, the cells initiate the production of, for example, inflammatory cytokines including IL-1, TNF-α, IFN-γ and IL-12. These cytokines activate natural killer (NK) cells and initiate a cascade of signals to cells of the adaptive immune response, preparing and

directing them for the development of antigen-specific immune responses. In this regard, molecules capable of stimulating innate immunity also act as adjuvants leading to enhanced antigen-specific immunity.

The induction of a Th1 response may be desirable for immunization against intracellular pathogens, whereas a Th2 response may be more important in protecting against helminthic parasites and for the induction of antibody-mediated immunity. Therefore, vaccines should be capable of selectively generating adaptive immune responses required for protection against the pathogen, while limiting the induction of inhibitory responses and side effects associated with excessive inflammation. Recent evidence suggests that certain adjuvants have the potential to selectively induce distinct T-cell subtypes by interacting with cells of the innate immune system. It is therefore vital to understand the factors that determine Th1 or Th2 differentiation in order to design better vaccines.

Adjuvants for mucosal vaccination: delivery systems and immune potentiators

Mucosal adjuvants are components which are coadministered with a vaccine to enhance the immunogenicity of vaccine antigens. Delivery systems allow vaccines to be successfully applied to mucosal surfaces and to promote their interaction with the MALT, and/or protect antigens from degradation, whereas immune potentiators specifically activate cells of the immune system.

In the following section we will briefly present delivery systems such as microparticles, chitosan, liposomes, immune-stimulating complexes (ISCOMs) and DNA vaccines. For a detailed discussion of the use of other delivery systems, such as live attenuated bacterial and viral vectors, readers are referred to recently published reviews (30-35). Furthermore, the use of immune potentiators such as bacterial toxins and their derivatives, cytokines, monophosphoryl lipid A and CpG motifs will be discussed.

Delivery systems

Microparticles

The potential of microparticles and other polymeric systems (36, 37), as well as the use of a broader range of antigen delivery systems (38), was recently reviewed. The polymers principally used in the microencapsulation of vaccines are aliphatic polyesters, poly(lactides) and poly(lactide-co-glycolides). It has been shown that the size of the microparticles, which can be controlled by the formulation conditions, is an important parameter determining efficacy (39). Antigens formulated with small particles (< 10 μm) are significantly more immunogenic than those formulated with larger particles. In *in vitro* studies, only particles smaller than about 100 nm have been demonstrated to be taken up by Caco-2 cells (40). However, it has been shown *in vivo* that microparticles up to 10 μm end up in Peyer's patches after uptake by M-cells (41, 42).

Oral immunization with the biodegradable and biocompatible poly(lactide-co-glycolide) (PLG) polymers has been shown to induce potent mucosal and systemic immunity to entrapped antigens (43, 44). These polymers have been used in humans for many years as surgical material and as controlled-release drug delivery systems, and are the primary candidates for the development of microparticles as vaccine delivery systems (45). The ability of microparticles to enhance immune responses to entrapped antigens following mucosal delivery is considered to be a consequence of their uptake into the specialized MALT (46), especially by M-cells, and the ability to target Peyer's patches in the intestine following oral administration.

By the intranasal route, microparticle-delivered antigens are able to activate the NALT and draining lymph nodes (47). A number of polymers, such as for example PLG, chitosan, starch, sodium alginate and hydrogels, have been used in mucosal vaccine administration, providing protective immunity in several animal models of infection, including *Streptococcus pneumoniae* (48, 49), *Salmonella typhimurium* (50), *Chlamydia trachomatis* (51) and *Bordetella pertussis* infections (52-54).

Oral immunization of mice with encapsulated malaria synthetic peptide SPf66 resulted in a Th1-like immune response (55). Immunization of mice with a microencapsulated recombinant antigen from *Mycobacterium tuberculosis* induced high levels of IFN-γ, indicating that microparticles preferentially induce Th1 responses (56). The potency of mucosal vaccines may be improved by the incorporation of lectins to target the vaccine more effectively to M-cells (57). Recent studies have shown that the efficacy of an *M. tuberculosis* protein-microsphere formulation was greatly enhanced by coencapsulation of the protein with monophosphoryl lipid A (MPL) adjuvant or the synthetic TLR4 agonist RC-529 (58).

Chitosan and derivatives

Chitosan, a linear polysaccharide, is prepared by deacetylation of chitin. As a mucosal delivery system, chitosan increases the bioavailability of the drug due to adhesion to mucosal surfaces and adsorption-enhancing effects. It also improves paracellular drug transport by opening intercellular tight junctions (59-61). It has also been suggested that chitin and chitosan may have immunomodulatory activities beyond antigen uptake at mucosal surfaces (62).

Chitosan is used in multiple pharmaceutical preparations (63, 64), has considerable potential as a mucosal vaccine delivery system (65-67), and has now been successfully tested for intranasal immunization with detoxified diphtheria toxin (DT) in healthy volunteers (68, 69). Furthermore, chitosan derivatives with superior solubility

at a broader pH range have been synthesized and evaluated (70, 71). Intranasal immunization with various vaccines formulated with chitosan or derivative induces neutralizing antibodies and Th2-type responses in mice (72, 73). Peroral vaccination with DT-loaded chitosan microparticles showed a strong enhancement of the systemic and local immune responses (74, 75). Oral administration of DNA nanoparticles, synthesized by complexing chitosan with plasmid DNA encoding food allergens, resulted in transduced gene expression in the intestinal epithelium of mice. Elevated secretory IgA and serum IgG_{2a} responses were measured; in addition, challenge studies in mice showed a substantial reduction in allergen-induced anaphylaxis associated with reduced levels of IgE and plasma histamine and vascular leakage (76).

Liposomes

Liposomes are bilayered membranes composed of amphipathic molecules (polar and nonpolar portions) such as lipids and cholesterol. Following uptake by macrophages and M-cells, they have the capacity to enhance the immunogenicity of mucosally delivered antigens which are entrapped, surface-linked or admixed. Liposomes can be prepared to vary in membrane stability, fluidity, size and permeability, depending on their lipid content and charge. Stability in acidic solutions, bile and pancreatin solutions, which demonstrates their suitability as oral vaccine delivery vehicles, was shown for several liposome formulations (77). Local IgA and IgG responses were induced by intranasal immunization with a liposome-formulated whole-cell *Yersinia pestis* vaccine, and protection against respiratory bacterial challenge was obtained (78). Nasal administration of the *Streptococcus mutans* antigen formulated in liposomes enhanced local secretory IgA in human volunteers (79, 80). Interestingly, conjugation of recombinant cholera toxin (CT) B subunit or CT to a liposome-*S. mutans* antigen preparation led to an enhanced mucosal immune response to the antigen incorporated in liposomes following oral immunization (81). Furthermore, immune responses to orally or nasally delivered liposome-formulated antigens were enhanced when MPL was incorporated in the liposome membrane (82). A recent study showed that intranasal administration of a synthetic peptide encapsulated in liposomes together with an anti-CD40 antibody induced protective immunity against influenza A virus in mice (83).

Immune-stimulating complexes (ISCOMs)

Immune-stimulating complexes, or ISCOMs, are cage-like structures consisting of cholesterol, phosphatidylcholine and the saponin adjuvant Quil-A. The typical ISCOMs are icosahedral structures of 40 nm in size and comprised of 10-12 smaller subunits (84). ISCOMs are preferentially taken up by nonspecific mechanisms, such as phagocytosis or micropinocytosis, by dendritic cells and perhaps by macrophages, whereas B-cells are probably unable to take up ISCOMs (85). An ISCOM without an antigen is known as an ISCOMATRIX (IMX) adjuvant, and whereas an ISCOM vaccine requires the antigen to be present during formulation, an IMX vaccine is made by simply mixing preformed adjuvant and antigen. Intranasal delivery of inactivated influenza vaccine plus IMX induced serum hemagglutination inhibition (HAI) titers higher than those induced by unadjuvanted vaccine delivered subcutaneously (86). High local and systemic antibody responses to respiratory syncytial virus (RSV) envelope antigens were induced after nasal delivery with ISCOMs (87). ISCOMs were also shown to augment local and systemic immune responses to antigens delivered by the oral route (88). They are effective at inducing T-cell responses, particularly $CD8^+$ cytotoxic T-lymphocytes (CTLs) and Th1 cells, as well as enhancing antigen uptake by APCs. This might reflect their ability to stimulate the production of IL-12 by cells of the innate immune system (89). Recently, ISCOMs were combined with a novel, nontoxic CTA-DD derivative into a vaccine adjuvant vector, resulting in the induction of strong cell-mediated and humoral immune responses (90).

DNA vaccines

DNA immunization is a very effective method for the induction of both humoral and cell-mediated immune responses. Together with easy manipulation and low production costs, this makes the development of DNA-based vaccines a very attractive alternative to attenuated or recombinant vaccines. The major drawback is the need for high doses to achieve good immune responses and protection in larger animals; in addition, their safety profiles remain unknown.

DNA vaccination has been attempted by intranasal or oral administration of a plasmid expression system for herpes simplex virus (HSV) (91-93), HIV delivered with microparticles (94), influenza virus (95, 96) and rotavirus (97), with significant levels of protection against mucosal challenge. A plasmid DNA vaccine encoding the circumsporozoite protein of the malarial parasite has been shown to elicit protective immunity against live sporozoite challenge in mice (98). In humans, the oral delivery of an HIV DNA vaccine induced local inflammation and activation of T-cells within the mucosa, but negligible antigen-specific T-cell and B-cell responses (99). Mucosal DNA vaccines are still in their infancy, and substantial improvements are needed to ensure efficacy in humans.

Edible vaccines

Stable integration of a gene into the plant nuclear or chloroplast genome can transform higher plants (*e.g.,* tobacco, potato, tomato, banana) into bioreactors for the production of subunit vaccines for oral administration (100, 101). The feasibility of generating recombinant

plants for the preparation of vaccine antigens has been demonstrated in tobacco plants, potato tubers and other edible plants. Potato tubers have been shown to successfully express several protein antigens for a number of human pathogens, including the *Escherichia coli* heat-labile enterotoxin (LT) B subunit (102), hepatitis B virus surface antigen (103), rotavirus (104), human papillomavirus-like particles (105) and measles (106). In a vaccination study in humans with Norwalk virus capsid protein assembled into virus-like particles, an immune response was obtained in 19 of the 20 volunteers, although there was only a modest increase in antibodies (107). Even if the level of protein expressed in the recombinant plant system appears to be variable and often low, the approach offers unique opportunities to develop vaccine strategies which can induce mucosal as well as systemic immune responses and can be delivered as part of a normal human biological function, *i.e.,* eating.

Immune potentiators

Bacterial toxins and derivatives

Cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) are the most powerful mucosal adjuvants known to date. Both CT and LT are ADP-ribosylating bacterial toxins that consist of an enzymatically active A subunit of 27 kDa and a pentameric B subunit of 55 kDa (108). The A subunit contains two domains. The A1 domain possesses ADP-ribosylating activity, which is responsible for the toxicity. The A2 domain interacts with B oligomer. The enzymatic activity requires the proteolytic cleavage of the loop between the two domains and the reduction of a disulfide bridge between A1-Cys^{187} and A2-Cys^{199}. The B subunit is an oligomer composed of five identical monomers, not covalently linked. It binds the receptors on the surface of eukaryotic cells.

Following the binding of LT to its receptor, the toxin is internalized into vesicles, transported to the golgi and disassembled. The A subunit translocates to the endoplasmic reticulum and then to the cytosol, where it interacts with ADP ribosylation factors (ARFs), enhancing its ADP-ribosyltransferase activity. The A1 subunit migrates to the plasma membrane, where it permanently activates the subunit of Gs, a GTP-binding protein, by ADP ribosylation (108). The outcome of this activation, which is the basis of the toxicity, is abnormal intracellular accumulation of cAMP, a subsequent increase in chloride and water secretion from the intestinal cells, and diarrhea.

The effect of LT on the immune system is distinct from that of CT. CT preferentially activates T-helper Th2-type $CD4^+$ cell populations, suppresses Th1 cell differentiation and induces immunoglobulin IgG_1, IgE and mucosal IgA (109). Conversely, LT does not show a marked preference for a Th population, activating both Th1- and Th2-type responses, and induces IgG_1, IgG_{2a}, IgG_{2b} and mucosal IgA (110, 111). It has been suggested that the polarization of the T-cell response may be driven by the A subunit. In fact, LTA-CTB and CTA-LTB, hybrids consisting of A and B subunits from the two different toxins, induce cytokine patterns similar to those induced by wild-type LT and CT, respectively (112).

Both CT and LT are potent mucosal adjuvants but are too toxic for clinical use (113). The observation that CT can redirect vaccine proteins to olfactory tissues (114) has raised safety concerns because GM_1-binding molecules might facilitate targeting of vaccines to neuronal tissues. Furthermore, an intranasal influenza vaccine containing LT as adjuvant was withdrawn from the market after an association with cases of Bell's palsy was demonstrated (115).

Thus, nontoxic or attenuated forms of CT and LT have been created by mutation. These mutants have also been shown to act as effective mucosal adjuvants (116-119). The nontoxic mutant LTK63 has been shown to enhance Th1 and Th2 responses to nasally delivered coadministered pertussis antigens, whereas the partially toxic mutant LTR72 enhanced Th2 responses (120). CT has proven to augment Th2 responses to orally delivered antigens in MALT (121) and to enable human dendritic cells to polarize precursor Th cells towards a Th2 phenotype (122). A recent study demonstrated that CT induces migration of dendritic cells from the subepithelial dome region to T- and B-cell areas of Peyer's patches (123).

Mucosal immunization with vaccines formulated with CT, LT or mutants as adjuvants provides effective protective immunity in several animal models of infection. For example, protection was conferred to mice against infection with *Helicobacter pylori* after oral immunization with recombinant vacuolating cytotoxin A (VacA), urease and cytotoxin-associated gene A (CagA) antigens formulated with LTK63 (124). Intranasal immunization with conjugate vaccines or protein subunit vaccines formulated with LT mutants protected against invasive pneumococcal (125) and *B. pertussis* (119) infections, respectively. Protection against pathology or infection has also been reported with *Schistosoma mansoni* (126) and HSV (127) antigens delivered by mucosal routes with CT as adjuvant.

Recent studies showed that the concomitant use of LTK63 mutant and delivery systems enhanced the immunogenicity and efficacy of a *Neisseria meningitidis* group C vaccine and further allowed a reduction in the amount of LTK63 needed, thus enhancing the safety profile (72, 128).

Cytokines

Most adjuvants exert some of their activity through the induction of inflammatory or Th1-inducing cytokines and chemokines, but they are often inappropriate for human use because of their unwanted side effects. An approach to circumvent this problem is to mimic the signals they induce *in vivo* by simply adding these signaling molecules either directly as proteins, or indirectly as coding DNA (129). During immune responses, cytokines play a role in the regulation of the selective induction and differentiation

of Th1 and Th2 cells. The adjuvant effect mediated by cytokines may function at the level of antigen presentation and/or the generation and maintenance of antigen-specific effector and memory T- and B-cells (130, 131).

IL-1 is a potent adjuvant when administered intranasally and was shown to enhance antigen-specific serum IgG, vaginal IgG and IgA, systemic delayed-type hypersensitivity and T-cell responses against coadministered antigens such as ovalbumin or tetanus toxoid (132). Intranasally delivered IL-6 and IL-12 enhanced antigen-specific serum antibody responses to a coadministered protein antigen, but only IL-12 was able to trigger antigen-specific secretory IgA responses (133, 134). Moreover, IL-12 has been shown to augment the Th1-type response when administered nasally with a tetanus toxoid and CT vaccine (135).

A strong antigen-specific Th1 response was induced after intranasal administration of a DNA vaccine encoding HIV antigens together with an IL-2 expression plasmid (136). Mucosal delivery of certain cytokines is also being assessed for the treatment of Th1-mediated inflammatory responses. Intranasal delivery of DNA encoding IL-10 resulted in diminished delayed-type hypersensitivity reactions in mice primed with HSV (137). Furthermore, combined nasal administration of autoantigen and IL-10 suppressed experimental allergic encephalomyelitis (EAE) in rats (138).

Nasal coadministration of the proinflammatory cytokines IL-1α, IL-12 and IL-18 induced antibodies against HIV peptide in serum and mucosal secretions and may replace CT as a mucosal adjuvant with HIV and other vaccines (139).

Monophosphoryl lipid A and other lipid A derivatives

Monophosphoryl lipid A (MPL) is a chemically modified derivative of the lipopolysaccharide (LPS) of Gram-negative bacteria (primarily from *Salmonella minnesota*), with reduced toxicity but potent immunomodulatory activity. Preliminary data indicate that the immunostimulatory activity of MPL is mediated via binding to the TLR4 complex, resulting in the production of proinflammatory cytokines. MPL induces the synthesis and release of IL-12 by dendritic cells and macrophages, and results in enhanced complement-fixing antibodies and the induction of antigen-specific cellular immunity (140, 141). MPL has been shown to enhance local and systemic antibody responses to influenza virus antigens (142) and to soluble or liposome-formulated *S. mutans* crude glucosyltransferase delivered by the nasal or oral route (82). Oral booster vaccinations with a combination of two efficient subunit *M. tuberculosis* vaccines with MPL as adjuvant protected animals against aerosol infection with *M. tuberculosis* (143).

Like MPLs, a new family of synthetic lipid A mimetics, the aminoalkyl glucosaminide phosphates (AGPs), also act on TLR4. These AGPs induce a classical TLR response, resulting in upregulation of costimulatory molecules, cell-surface receptors, cytokines and chemokines. The AGPs (for example, RC-529) also possess potent adjuvant activity with mucosally delivered vaccines (144).

CpG motifs of bacterial DNA

As bacterial DNA is able to activate immune cells, synthetic oligodeoxynucleotides (ODNs) containing unmethylated deoxycytidyl-deoxyguanosine (CpG) dinucleotides, usually flanked by two 5'-purines and two 3'-pyrimidines at either end of the motif sequence (CpG motifs), stimulate the innate immune system to induce protective responses in mice and primates (145-147). This motif is approximately 20 times less common in mammalian DNA than in bacterial DNA (148). Synthetic ODNs containing immunostimulatory CpG motifs can activate human B-cells, dendritic cells and NK cells (149). They can trigger an immune cascade that includes the production of cytokines such as IL-12, TNF-α and IFN-γ, and are extremely efficient inducers of Th1 immunity and CTLs (150, 151). Interactions between CpG-ODNs and TLR9 receptors in APCs, such as dendritic cells and macrophages, rapidly stimulate, through the Toll/IL-1 receptor signaling pathway, the cells to produce proinflammatory and Th1-polarizing cytokines, such as IFN-γ, IL-1β and IL-12, to upregulate costimulatory molecules on the APCs and to activate B-cells for proliferation, antibody production and IL-6 secretion (152).

CpG-ODNs were able to enhance local and systemic antibody responses to oral, intrarectal or intranasal immunization with tetanus toxoid or influenza virus vaccines (153). Interestingly, "control" non-CpG-ODNs also had adjuvant effects when used mucosally, but selectively induced a Th2 immune response. The same group showed that humoral and cell-mediated systemic and mucosal responses are induced after intranasal immunization of mice with hepatitis B surface antigen given together with CpG DNA (154). CpG-ODNs markedly increased the humoral neutralizing antibody response to RSV after nasal administration in rats (155). Furthermore, the efficacy of CpG as a potent mucosal adjuvant has been demonstrated in mucosal immunization studies with *Mycobacterium bovis* BCG (156), rotavirus (157), inactivated HIV (158), *Haemophilus influenzae* type b conjugate vaccine (159), a synthetic measles virus peptide (160) and HSV (161).

Importantly, the formulation of CpG-ODNs in appropriate lipid-based delivery systems constitutes a means of potentiating the adjuvant effect of these immunostimulatory molecules (162). However, a recent study showed that oral immunization against *H. pylori* with CpG as adjuvant neither induced sterilizing immunity nor led to complete protection from disease (163).

Conclusions

Despite the attractiveness of mucosal immunization, only a few mucosal vaccines are available at the present

time, all of which are live attenuated vaccines. No mucosal vaccines consisting of defined proteins have yet been prepared.

The overall experience with these licensed oral vaccines, including the Sabin live attenuated oral polio vaccine (OPV) and typhoid vaccine (164), is extremely positive. Other examples of mucosal vaccines are a live cholera vaccine (165), a nonlive cholera vaccine (166, 167), a live BCG vaccine and an oral adenovirus vaccine. However, since poliomyelitis has been eradicated in North America, the OPV is no longer recommended for routine use in the United States, and the other oral vaccines mentioned above are not available for routine use. Furthermore, a tetravalent reassortant rhesus rotavirus vaccine (168) and an intranasal nonlive influenza vaccine adjuvanted with LT (115) were withdrawn from the market because of severe side effects, emphasizing the difficult and challenging task of combining vaccine and adjuvant efficacy with safety.

Very recently, a cold-adapted, live attenuated, trivalent intranasal influenza virus vaccine was approved by the U.S. Food and Drug Administration in 2003 and has been shown to be safe and effective, although its role in the prevention of influenza has yet to be defined (169).

As mentioned above, mucosally delivered proteins require strong adjuvants to be immunogenic. The development of new adjuvants is essentially hampered by safety concerns, lack of universality and lack of suitable animal models. The current attitude regarding the risk-benefit ratio of vaccination places a large emphasis on safety since vaccines are given to healthy individuals.

Advances in the design of more efficient mucosal vaccine delivery systems and technologies and more potent immune modulators that can efficiently help to direct the vaccine antigens to the mucosal immune system have been instrumental in identifying new vaccine formulations that are safe and effective when delivered by mucosal routes. Furthermore, the possibility of directing the immune response to humoral or cell-mediated immunity and selectively inducing or balancing Th1- or Th2-type responses, favors the design of vaccines based on specific knowledge of the protective mechanisms.

Hopefully, continuing advances in the understanding of the immune system will enable the development of better vaccines. This together with progress in the pharmaceutical formulation of appropriate delivery systems will enable the design of mucosal vaccines better suited to health needs worldwide.

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