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MUCOSAL DELIVERY OF VACCINES: A REVIEW

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Abstract:

Mucosal surfaces of the gastrointestinal, urogenital and respiratory tracts are a common site of entry for many human pathogens that are a cause of infectious diseases globally. Vaccines have the ability to induce mucosal immune responses that can provide prophylactic and therapeutic responses against various diseases as well as cancer. Most licensed vaccines are administered via parenteral route and are unable to elicit protective mucosal immunity. Therefore, mucosal routes for immunization may be more favourable in providing protective immunity against mucosal pathogens at the portals of entry. However, a number of challenges are associated with different types of vaccines. The present review summarizes the various delivery strategies that can improve the mucosal delivery of the vaccines, the carrier systems and the adjuvants that can be used along with the vaccines to confront the hurdles of design of mucosal vaccines, thereby enhancing mucosal vaccination. Among the different approaches for effective mucosal vaccination, particulate delivery systems can be employed successfully by protecting the immunogenic material during the delivery, facilitating specific target oriented delivery and by allowing incorporation of several adjuvant materials. Efficient delivery of vaccine antigens to mucosal sites that assist in uptake by local antigen-presenting cells to generate protective mucosal immune response has also been addressed. The last part of the review points out the future issues regarding mucosal vaccine development that includes targeting mucosal dendritic cells as an effective and safe strategy for inducing antigen-specific immunity as well as finding out new routes of mucosal immunization and combination of appropriate delivery strategies.

Keywords: Mucosal delivery, Vaccines, Adjuvants, Particulate delivery system.

1. Introduction

Mucosal surfaces are large surface areas that are a common site of entry for pathogenic microorganisms [1]. The

presence of antigens, pathogens and vaccines within the body that enter through mucosal surfaces are easily detected by the adaptive immune system from those that are introduced directly into tissues or the bloodstream by injection or injury [2]. This clearly indicates that effective mucosal vaccines (oral, nasal, sublingual and genital tract vaccines) can significantly contribute to the improvement of global heath as they have the capability to stimulate protective immune responses not only against mucosal infections but also against HIV and also by producing mucosal antibodies against *Vibrio cholerae* bacteria and cholera toxin that is associated with resistance to cholera and many other infections [2,3].

The vaccines that are administered onto the mucosal surfaces offer a number of advantages over injected vaccines from a production and regulatory viewpoint [4,5]. Oral vaccines do not require rigorous purification from bacterial by-products owing to the presence of normal microbiota in the human gut whereas the same formulation of vaccine injected through parenteral route will result in unacceptable levels of endotoxin. Moreover, mucosal vaccines are more feasible for mass vaccination due to needle-free administration, and prevent the risk of spreading blood-borne infections that is more likely to occur with the use of contaminated injection needles. Other benefits of mucosal vaccines include ease of administration, better patient compliance and the greater probability of delivering the vaccines by personnel with minimal training especially for preventing the spread of infections worldwide such as influenza virus infections [6–8]. A number of mucosal vaccines that have been approved for human use that include oral vaccines against poliovirus, *Salmonella typhi*, *V. cholerae* and rotavirus, and a nasal vaccine against influenza virus (Tradename: FluMist) [3].

However, mucosal delivery of vaccines presents a number of challenges in terms of dosing and formulation strategies. An ideal target-delivered dose of vaccine cannot be easily determined and booster doses may be required to induce sufficient protective immunity.

The need for identifying the required dose and timing of booster shots makes mucosal vaccination complicated [9]. Measurement of mucosal immune responses is usually more complex since the dose of mucosal vaccine that actually enters the body cannot be actually measured. The antibodies produced as a result of the mucosal vaccines are difficult to capture and quantitate and recovery and functional testing of mucosal T cells is not only labour intensive but also technically challenging [1]. Furthermore, prior mucosal exposure to antigens increases the risk of inducing mucosal tolerance instead of protective immunity that may reduce the effectiveness of mucosal vaccines and hence adjuvants

need to be incorporated in formulation of mucosal vaccines to stimulate stronger immune responses [3].

The present review summarizes the various delivery strategies that can improve the mucosal delivery of the vaccines, the carrier systems and the adjuvants that can be used along with the vaccines to confront the hurdles of design of mucosal vaccines, thereby enhancing mucosal vaccination. The last part of the review points out the future issues regarding mucosal vaccine development as well as finding out new routes of mucosal immunization and antigen delivery systems along with novel mucosal adjuvants.

2. Mucosal Immune System

The mucosal membrane is a strong component of the immune system that covers the eye conjunctiva, the inner ear, the digestive and the urogenital tracts, the respiratory canal and layers of most of the exocrine glands [10]. The three main functions of mucosal immune system are (i) to protect the mucous membranes against colonization and invasion of microbes, (ii) to prevent uptake of undegraded antigens including foreign proteins that are derived from ingested food, airborne matter and commensal microorganisms and (iii) to prevent potentially harmful immune responses that may occur if these antigens enter the body [11].

The mucosal immune system constitutes an integrated network of tissues, lymphoid and nonlymphoid cells, and effector molecules such as antibodies, chemokines and cytokines [12] [9]. The mucosa is a local and but more specialized version of the body's immune system and well associated with the lymphatic system and hence it is known as the mucosa associated lymphatic tissue (MALT), and to be more precise in terms of its functioning, it is called the mucosal immune system. The mucosa associated lymphatic tissue is a wider term and it consists of many different subdivisions namely the bronchus associated lymphatic tissue (BALT), the gut-associated lymphatic tissue (GALT), and the nasal associated lymphatic tissue (NALT) [10]. The initiation of the antigen-immune response occurs in MALT [9].

The organization of cells and tissues of the mucosal immune system differs from that of the systemic immune system and understanding these differences is imperative for the rational design of prophylactic vaccines to protect against mucosal infections [9]. The mucosal surfaces can be classified as Type I and Type II mucosae. Type I mucosae are represented by the surfaces of the lung and gut, whereas Type II mucosae include the surfaces of the mouth, esophagus and cornea. The female genital tract has both Type I (endocervix, uterus) and Type II (vagina, ectocervix) mucosae.

The surfaces of Type I mucosae are covered by simple columnar epithelium linked by tight junctions whereas Type II mucosae are lined with stratified squamous epithelium. MALT is present beneath the Type I epithelial layer [13]. Epithelial microfold cells (M cells) covering the MALT play a crucial role in antigen delivery across the epithelial barrier as they do not possess an organized brush border or cilia and have a basolateral pocket that facilitates direct contact with B cells and $CD4^+$ T cells, therefore can trigger mucosal immune responses, making them an ideal target for mucosal vaccine delivery [9,14].

Type I mucosal epithelia expresses polymeric immunoglobulin receptor (pIgR) on the basolateral surfaces which binds to dimeric immunoglobulin A secreted by plasma cells in the lamina propria which is the connective tissue directly underlying the mucosal epithelium [13,15]. Secretory IgA (sIgA) is the main protective immunoglobulin in type I mucosal tissues and is a critical component of mucosal effector function. On the contrary, the main protective immunoglobulin of type II mucosal tissue is immunoglobulin G (IgG). Dendritic cells are present throughout the MALT and acts as antigen presenting cells locally or migrate to draining lymph nodes [9]. The mucosal immune system has two parts: the innate system and the adaptive system. The innate system consists of various recognition molecules and the natural killer cells whereas the adaptive system comprises of various antigen–presenting cells and T and B lymphocytes [10].

2.1 Mucosal Innate Immunity

The innate immune system is responsible for the initial systemic immune responses [9]. Foreign entities are recognized at the mucosal surfaces in a number of ways. Dendritic cells can identify cytosolic pathogen- associated microbial patterns (PAMPs) such as bacterial cell wall components (e.g peptidoglycan, lipoteichoic acid) and uncommon forms of nucleic acids (e.g double stranded RNA, high-CpG-content DNA) using various pattern recognition receptors such as Toll-like receptors (TLRs), followed by the presentation of pathogens, either by the major histocompatibility complex (MHC) Class I pathway (via rough endoplasmic reticulum) or the MHC Class II pathway (via autophagy). Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) induce costimulatory molecules and cytokines that activate T cells.

In cell-intrinsic recognition, the MHC I pathway depends on increased number of antigens whereas the MHC II pathway relies on the mechanism of autophagy. Cell-extrinsic recognition can recognize either pathogenic or infected

cells. When pattern recognition receptors encounter an infected cell, they induce secretion of interferons and dendritic cell-activating cytokines which is followed by MHC Class I or II antigen presentation. If Toll-like receptors are involved in recognition, they stimulate the production of costimulatory molecules and cytokines, thus activating the T cell response.

Pathogen toll-like receptors recognize the pathogen and move, along with the pathogen, into the cell phagosome where antigen processing and presentation occurs only through MHC Class II pathway, followed by production of T cell activation molecules (Figure 1) [16,17]. For mucosal vaccine design, the aim is to induce an adequate innate immune response for initiating adaptive immunity without causing excess inflammation, tissue damage or other types of sequelae [9].

2.2 Mucosal Adaptive Immunity

A characteristic feature of mucosal adaptive immunity is the local production and secretion of immunoglobulin A (IgA). Unlike other antibody isotypes, IgA are resistant to degradation in the protease-rich external environment of mucosal surfaces owing to its dimerization and its associated with a glycosylated fragment (the secretory component) which is derived from the epithelial polymeric immunoglobulin receptor (pIgR) that mediates transport of dimeric IgA across epithelial cells to the lumen [1,15]. Secretory IgA entraps antigens or microorganisms in the mucus, thus preventing direct contact of pathogens with the mucosal surface, a phenomenon known as 'immune exclusion'. They can also obstruct the microbial surface molecules that mediate epithelial attachment or may hinder the incoming pathogens within epithelial-cell vesicular compartments during pIgR-mediated transport [18].

The adaptive immune responses are mediated by B and T lymphocytes that utilize antigen receptors which are clonally distributed and produced through rearrangement of antigen receptor gene segments in the genome. The lymphocytes with antigen specific receptors undergo progressive multiplication and provide enhanced memory responses to the same antigen after repeated exposures [9]. The proportion of B cells to T cells is higher in organized mucosal lymphoid tissue in comparison to that found in peripheral tissues. Therefore the B cell responses in the intestine are more dependent on T cell-independent B-1 cells that are more active against polymeric antigens such as bacterial capsules and polymerized flagellin [19]. Thus, it is evident that targeting both T cell-independent and T cell-dependent B cells in the lymphoid tissues can enhance mucosal vaccination [9].

3. Hurdles of Mucosal Vaccine Design

Mucosal vaccines that are given orally or deposited directly on mucosal surfaces encounter the same host defences that microbial pathogens face. A major obstacle to mucosal immunization is that the mucosal vaccines are diluted in mucosal secretions and bulk flow may hinder the effective deposition onto the epithelium of the mucosal system. In addition, the mucosal vaccines get captured in mucus gels and are subsequently degraded by proteases and nucleases. Therefore the dose of vaccine required is relatively large and the exact dose that crosses the mucosa cannot be determined [1,20].

Mucosal vaccines that mimic the physicochemical properties (as for example, charge and size) of pathogens are likely to be more effective. There are various strategies for effective mucosal immunization that focuses on (i) overcoming physiological barriers at mucosal routes, (ii) targeting mucosal epithelial cells (M cells), antigen presenting cells (APC) (especially dendritic cells) for appropriate processing of antigens that lead to activation of B and T cells and (iii) efficiently stimulate innate responses to trigger adaptive immune responses appropriate for the target pathogen. The challenge for designing mucosal vaccines is to promote an enhanced immune response without compromising safety [1,20].

4. Delivery Systems for Mucosal Vaccines

There a number of approaches for designing delivery systems for mucosal vaccines. One such approach is to develop vaccines using either live-attenuated or inactivated infectious viral or bacterial pathogens. Another approach is to develop subunit vaccines by using recombinant viral or bacterial proteins that can induce sufficient immune response without causing adverse effects. The most promising delivery system for mucosal vaccines is based on synthetic particulate delivery system that is designed to imitate the immunogenic properties of pathogens [9]. These delivery systems are discussed in detail in the following sections.

4.1 Live-attenuated or Inactivated Vaccines

Vaccines based on live attenuated viruses or microbes consist of live bacteria or viruses that are made less virulent than the parental pathogenic bacterial or viral strains through inactivation by heat or chemicals. The characteristic feature of live attenuated vaccines is that they provide a high level of antigen exposure and have in-built adjuvanticity [21]. Live bacteria and viruses can function as vaccine vectors as they have the ability to carry recombinantly expressed antigens from other pathogens by the process of genetic engineering [3]. Several live attenuated mucosal vaccine vectors,

including poliovirus, adenovirus and enteric bacteria are currently undergoing development. The advantage of live attenuated pathogens as mucosal vaccines and vaccine vectors is partly due to their ability to activate multiple innate responses which in turn orchestrate adaptive immune responses [2,22]. However, vaccine vectors that express recombinant antigens may show lower vaccine efficacy compared to live attenuated vaccines due to the presence of pre-existing vector-specific immunity [23].

The live attenuated vaccines usually produce a mild infection at the site of administration. They can also be engineered to replicate in a confined area at the site of immunization in order to deliver a sufficiently high antigen load, resulting in a more effective mucosal response and thus avoiding undesirable local inflammatory responses [24]. The challenge associated with the development of live attenuated vaccines is to achieve a balance between sufficient attenuation and immunogenicity of vaccines.

For example, the live attenuated vaccines against rotavirus infection has been made less virulent and more effective by passing the virus serially in host cell cultures. These attenuated vaccines produced mild subclinical infection but were still highly immunogenic [24,25]. On the contrary, the live oral Ty21a typhoid vaccine has moderate immunogenicity but is rendered safe due to excessive attenuation. Nevertheless, the molecular basis for attenuation of these vaccines still needs to be elucidated [24,26]. The new generation live *S. typhi* vaccine, designed to be administered in a single dose has been attenuated by applying selected gene deletion that produced a safe vaccine with high immunogenicity [24,27].

Precise gene deletion is a more preferable strategy than serial passage for attenuation of live vaccines and for designing stable and safe live vaccines and thus demands a deeper understanding of the genetic basis for attenuation [3]. Many live attenuated vaccines are under clinical trials and that includes oral vaccines against *Shigella* spp. (the causative agents of shigellosis) and *Salmonella* infections, intranasal and sublingual vaccines against *Bordetella pertussis* (the causative agent of whooping cough), influenza virus, rotavirus, norovirus and measles virus [28–30].

4.2 Nonliving Whole-cell and Subunit / Conjugate Vaccines

In spite of the advantages of attenuated vaccines for their high immunogenicity and ability to deliver high levels of antigen to the mucosal target site, the development of these vaccines involves multiple steps that cannot always produce safe and stable vaccines. For this reason, the need for nonliving whole-cell or subunit/conjugate vaccines arises [3]. Sub-component vaccines based on pathogen-specific proteins or polysaccharides conjugated to proteins or peptides belong to

the second largest group of licensed prophylactic vaccines [9]. The only example of licensed non-living mucosal vaccine for human use is the oral cholera vaccine, named Dukoral, which was developed in the 1970s and the early 1980s. The vaccine consists of killed whole *Vibrio cholerae* bacteria conjugated with recombinant cholera toxin subunit B [31,32]. It offers protection even 2-3 years after vaccination and secretory IgA is mainly responsible for the resultant protective immune response to inhibit bacterial colonization and toxin binding to intestinal epithelial cells [33–35].

Several studies have shown the promise of subunit vaccines as an approach for mucosal vaccine development. A subunit vaccine composed of HSV-2 envelope glycoprotein fused to the IgG Fc fragment was developed for intranasal immunization which elicited mucosal B and T cell responses to give protection from HSV-2 [36]. A recent interesting prospect of subunit vaccines focuses on the use of proteins with defined structures as scaffolds for presenting immunogenic epitopes. An epitope scaffold vaccine has been prepared for HIV-1 envelope protein gp41epitopes and has produced a significant number of antibodies [37–39]. However, there are challenges associated with the development of subunit vaccines for mucosal immunization. The protein antigens are subjected to degradation by the mucosal proteases or commensal microbiota and therefore needs to be protected. Moreover, the subunit vaccines have low immunogenicity and require the use of adjuvants to produce stronger immune response. The route of immunization needs to be carefully chosen as well [9].

4.3 Particulate Delivery Systems

Particulate delivery systems can be used to enhance the efficacy of mucosal vaccines. The particle – mediated delivery systems can protect the vaccines from degradation by extracellular enzymes and various extreme pH environments, improve targeted cellular uptake and subsequent endosomal release [40,41]. The size, shape, material and characteristics of the particle delivery system must be taken into consideration. Particle size is a critical factor as particle endocytosis by the various mucosal phagocytotic cells activates mucosal immune system. The phagocytotic cells can ingest particles within the size range of 1 and 5µm whereas the non- phagocytotic cells can only internalize the particles in the nanometer-size range. Another important factor that has a significant effect on phagocytosis is the shape of the particles. Spherical and cylindrical shaped particles tend to be ingested more compared with disk-shaped particles [42]. The carrier system should be biodegradable, biocompatible with a high loading capacity and a positive charge if cell membrane interaction is required [40]. The different types of particulate delivery systems are discussed here.

4.3.1 Virus-like Particles and Virosomes

Virus-like particles (VLPs) are self-assembling, nonreplicating viral core structures, usually from non-enveloped viruses that are formed by spontaneous assembly of recombinant viral proteins *in vitro* or by budding from transfected cells in culture [1,11]. They have native viral surface structures but lack nucleic acids and are therefore unable to replicate in cells or cause infection [1]. VLPs are cheap, easy to prepare and are highly immunogenic and are therefore of commercial interest as viral vaccines. They can also serve as combined carriers and adjuvants for foreign antigens that are expressed recombinantly on their surface as well as for DNA vaccines incorporated within VLPs. VLPs are particularly promising for the design of mucosal vaccines as they offer the prospect for the natural route of transmission of the parent virus to be used for delivery of vaccines and produces sufficient amount of secretory IgA and cytotoxic T cells, thus resulting in greater mucosal immune response (Holmgren paper). The first commercially viable vaccine was the hepatitis B vaccine that was produced from the self-assembly of the hepatitis B surface antigen (HBsAg) expressed recombinantly in yeast cells. The other VLP licensed for human use is the human papillomavirus (HPV) vaccine. The quadrivalent HPV vaccine (Gardasil, Merck & Co.) is composed of the L1 capsid proteins of HPV-6,-11, -16 and -18 types that are expressed recombinantly in yeast and self-assembled into VLPs [9,43].

Virosomes are a special class of liposome vaccine delivery systems where the viral membrane proteins are integrated into unilamellar vesicles composed of viral and other natural or synthetic lipids [44]. The lipids used in virosomes can be derived from the virus, egg or synthetic lipids [45]. The most advanced type of virosomes are immunopotentiating reconstituted influenza virosomes (IRIVs). The presence of the influenza-derived proteins haemagglutinin and neuraminidase distinguishes the IRIVs from the liposomes. Examples of virosome vaccines include Epaxal (Crucell, Netherlands) for hepatitis A in which formalin-inactivated hepatitis A virus is adsorbed onto IRIVs and Inflexal (Crucell, Netherlands) which is a trivalent influenza vaccine composed of three monovalent virosomes reconstituted from different influenza virus strains [45–47]. Even though VLPs and virosomes have great potential as vaccine carriers owing to their small size that makes it appropriate for uptake by M cells and dendritic cells, the composition of their surface chemistry that mimics the mucosal pathogens, scope for incorporation of costimulatory and immunoregulatory proteins, yet they are difficult to formulate and are less reproducible compared to synthetic polymer based nanocapsules and nanoparticles [1,9].

4.3.2 Nonviral Polymer-Based Carrier Systems

Polymer-based micro and nanocarrier systems have proved to be successful in designing mucosal vaccines. The carriers may be natural or synthetic polymers, lipids, proteins that can be used to form particles and capsules of desired size and shape. The two most widely used nonviral polymer-based carrier systems are nanocapsules and nanoparticles. Nanocapsules consist of a reservoir delivery system in which the vaccine is enclosed within an aqueous or oil-based core that is covered by a solid or semisolid material shell. On the contrary, nanoparticles are solid drug delivery systems that adsorb, attach, dissolve or disperse a drug in a carrier matrix or contain the drug in an encapsulated system [9,42].

4.3.2.1 Nanocapsules

The two types of nonpolymeric carrier systems that have been approved for human use and have shown great promise as mucosal vaccines are emulsions and liposomes [9]. Emulsions are lipid based drug delivery system that consists of aqueous droplets dispersed in an oily medium or oil droplets dispersed in an aqueous environment and these emulsions are stabilized by surfactants such as Span 80 (oil soluble surfactant) and Tween 80 (water soluble surfactant) [48]. Nanoemulsions exhibit long-term colloidal stability and have been used to encapsulate and deliver vaccines directly onto the mucosal surfaces. The size range of nanoemulsions is from 20nm to 200nm which is close to the size of opportunistic pathogens and are readily taken up by mucosal M cells and subsequently presented to antigen presenting cells (APC). Hydrophilic drugs can be incorporated using water-in-oil emulsions whereas hydrophobic drugs can be incorporated in oil-in-water emulsions for efficient drug delivery [49].

The use of single-nanoemulsion technology has led to the production of hepatitis B vaccines. Recombinant HBsAg was successfully emulsified into uniform droplets and was delivered into mucosal effector sites intranasally which resulted in a stronger immune response, producing high titers of both IgA and IgG. This is also indicates that nanoemulsion can be employed for NALT mucosal immunization [50]. However, single-nanoemulsion methods show poor controlled release profiles and may not be able to withstand degradation within mucosal sites other than NALT (Reference 100 from Woodrow paper). Therefore the concept of a double emulsion method with good controlled release profiles was introduced by Hanson et al.[51]. Double-emulsions can be produced to carry both polar and nonpolar payloads and can be stabilized with synthetic amphiphilic diblock copolypeptide surfactants and droplet size can be reduced to below 100nm. This type of emulsions are more stable and are able to encapsulate antigens without any deleterious effect to the

antigens during the emulsification process and thus can be employed for efficient delivery of vaccines to mucosal surfaces before they are degraded [51].

Liposomes are one of the nonpolymeric drug carriers that has shown great potential as carriers for vaccine in mucosal immunization. They are made up of various phospholipid molecules that are based on the structure of natural biological membrane lipids. Liposomes being poorly water soluble, self-assemble into a phospholipid bilayer that can constitute into a multilamellar or unilamellar vesicle that surrounds an aqueous environment [52]. The hydrophobic bilayer and the aqueous core of the liposome are convenient for conveying lipophilic or hydrophilic compounds, respectively [53]. Liposomes can be made into small (1-μm) unilamellar vesicles [54,55]. Lipids can be specifically selected to tailor for specific function.

For instance, cationic lipids can be tailor-made for complexation and efficient delivery of nucleic acids, and pH-titratable lipids can be tailor-made for pH-triggered release of agents [53,56,57]. Moreover, liposomes can be readily surface modified with ligands for tissue and cell targeting, steric stabilization, mucoadhesion, and mucus penetration. Although considered inert, certain lipid constituents in the lipid matrix may cause inflammation. Therefore lipid compositions must be selected carefully for mucosal delivery.

Liposomes of different composition, size, and function can be prepared which gives them versatility as carriers for mucosal vaccines. Liposomes have been reported to have shown promising results for mucosal immunization for many groups. Liposome-based mucosal vaccines have been used mainly for oral or intranasal immunization. Rosada et al. [58] have shown that a single intranasal immunization with cationic liposomes that delivered a DNA encoding for a tuberculosis heat-shock protein was able to give protection against the bacterium by inducing strong cellular immune responses. Liposomes have undergone fabrication using immunomodulatory lipids such as polycationic sphingolipids or cationic cholesterol derivatives that can show adjuvanting activity upon mucosal administration [59,60]. Recently, an interbilayer, cross-linked multilamellar vesicle was used to codeliver antigen and adjuvant [61]. These stabilized liposome vaccines boosted humoral and cellular immune responses 10–1,000-times compared to the responses caused by soluble antigen alone or non-cross-linked multilamellar vesicles. These novel lipid systems which were used as subcutaneous vaccines possess potential for applications as mucosal vaccines. A huge number of preclinical studies have been performed testing the efficacy of liposomes as mucosal vaccines; however, no products have still been approved for

clinical use. Further research studies need to be carried out to explore on how the lipid matrix can be engineered so that it interacts effectively with the mucosal immune system.

4.3.2.2 Nanoparticles

The availability of different types of polymers and the various methods for particle synthesis increases the scope for using nanoparticles such as micelles, dendrimers and solid matrix nanoparticles in designing mucosal vaccines. The polymers used can be of natural or synthetic origin. Chitosan, alginate, albumin are examples of natural polymers and polyesters, polyanhydrides, poly(amino acids) can be used as synthetic polymers. The particulate delivery systems based on synthetic or natural biodegradable polymers enable the control of timing of the antigens as well as adjuvant presentation. The formulation of nanoparticles employs different emulsification techniques that induce polymer precipitation as a result of solvent removal by extraction, evaporation, diffusion or de-salting [62]. Nanoparticles can also be formed through gelation of polymers that have gelling properties in response to temperature, pH or presence of cross-linking agents which are dispersed in emulsion droplets [63]. One of the most widely used copolymers for drug delivery is poly(D,L-lactide-co-glycolide) (PLGA) because it is safe to use and has minimum toxic effects with controlled release property [64]. PLGA nanoparticles can be modified by encapsulating the vaccine antigens into the matrix or on the surface of the nanoparticles to tailor the physicochemical properties that will enhance the permeation of the particles through the mucosa and transcytosis by the M cells [65]. Polymeric nanoparticles also use certain types of ligands for effective targeting and uptake by mucosal epithelia and APCs. For example, nanoparticles comprising of a PLGA core and surface modified with a PEG lipid for delivery of a humanized antibody were targeted at human dendritic cells [66].

The major factors that determine the amount of antigen delivered and internalization of the antigen are size and surface chemistry. The polymeric micelles and dendrimers can be designed in the ultrasmall size range (<25nm). Polymeric micelles are formed as a result of self-assembling of amphiphilic di- or tri- block copolymers into spherical nanosized core/shell structure in aqueous media**.** The hydrophilic and hydrophobic parts of the polymeric micelles allow encapsulation of the delivery agent within the core or attached to the polymer shell [67]. However these polymeric micelles are subjected to dissociation upon dilution that can result in undesirable and abrupt release of the targeted agent. In this context, dendrimers offer greater stability compared to polymeric micelles due to the presence of covalent bonds that is responsible for the branched polymer network [67]. Specific engineering of the surface chemistry and polymer

composition of the nanoparticles can be utilized to overcome the mucosal barriers and promote immunomodulatory function at mucosal sites. The hydrophilic and hydrophobic segments can change the microstructure of the mucus and promote mucoadhesion and mucus penetration. Functional groups and surface ligands can also be attached to the nanoparticles for activation of the complement system and to mimic PAMPs [67].

5. The Need for Mucosal Adjuvants

Vaccines may not sufficiently elicit a strong immune response even after successful delivery of the antigen and immune system activation. However an antigen-induced immune response can be improved by adding substances known as adjuvants. Vaccine adjuvants work by protecting the antigen, regulating cytokine release, triggering $CD8^+$ CTL responses or assisting in antigen delivery to the target tissue [68] . An antigenic protein, carbohydrate or lipopolysaccharide additive can work as adjuvant when administered along with the existing attenuated vaccine [69,70]. For attaining protection against intracellular pathogens and viruses, the initiation of CTL and Th1 T-cell responses is required. However antigenic peptides incorporated on the surfaces of delivery vehicles are not able to induce these responses in mucosal vaccine systems adequately [71]. Hence specific and enhanced mucosal adjuvants are required which can safely activate cellular and humoral immune responses with the desired Th cell type profile to bring out both mucosal and systemic protection [72].

Mucosal adjuvants include lipopolysaccharide (LPS)-protein complexes (endotoxins), monophosphoryl lipid A (MPL), polyribonucleotide complexes, imiquimod, muramyl dipeptide and analogues, nonionic block copolymers, saponins (ISCOMS), the oral mucosal adjuvant cholera enterotoxin (CT) and dehydroepiandrosterone (DHEA) [73]. Cholera toxin (CT), an exotoxin produced by *Vibrio cholerae* is a mucosal adjuvant that induces enhancement of antigen-specific mucosal IgA and systemic IgG responses to proteins administered mucosally [74]. DHEA is a steroid hormone that boosts cell-mediated immunity, which is essential for protection from intracellular pathogens and viruses [73]. CpG motifs in synthetic oligodeoxynucleotides have shown to work as oral adjuvants bringing about the activation of Th1 type directed immune response [75]. These sequences when incorporated into therapeutic genetic material can initiate B-cells to proliferate and secrete immunoglobulins, activate APCs and trigger cytokine production [76].

6. Routes of Mucosal Immunization

Various routes of mucosal immunization have been developed for targeting MALT and have proved to elicit protective

mucosal immunity against several pathogens successfully. The route of immunization, the adjuvant and the method of antigen delivery must be carefully considered to produce the desired mucosal immune responses to a particular pathogen [77]. Oral vaccinations result in fewer side effects and may be considered as the most preferred route of immunization because of ease of administration. However, oral vaccines are likely to be exposed to the harsh environment of the gastrointestinal tract and therefore it is imperative to maintain their original quality and efficacy until they reach the GALT [77]. In this context, rice based vaccines can provide an effective strategy for the development of cold chain and needle-free vaccination that can protect from gastrointestinal infection and shows greater stability at room temperature for 2-3 years.

An example of such a vaccine is MucoRice-cholera toxin B-subunit that did not lose activity upon exposure to digestive enzymes and played a critical role in protection against cholera toxin induced diarrhea [78].

Nasal route of administration has shown particular potential in providing both humoral and cell-mediated responses at the particular mucosal surface and also throughout the body. Vaccines delivered by nasal route has induced several mucosal IgA antibody responses in the salivary glands, upper and lower respiratory tracts, male and female genital tracts and the small and large intestines in mice, monkeys and humans [79–81]. Moreover, nasal immunization studies in humans and mice have generated greater systemic antibody responses in comparison to other routes of mucosal immunization [1].

Although oral and nasal routes are the most preferred mucosal immunization routes, rectal and vaginal immunization routes can also be used for inducing protective immunity against sexually transmitted diseases, including HIV [82–85]. In addition to these routes, sublingual route of immunization can be utilized to treat allergic, autoimmune or infection-induced pathologic reactions [11,86]. Similar to sublingual route of immunization, eye drop immunization can efficiently result in mucosal immunity without causing serious toxicity or adverse effects. However, the exact cellular and molecular mechanisms of these immunization systems for inducing mucosal immunity yet remain to be elucidated. Nevertheless, appropriate combination of mucosal adjuvants and delivery systems and optimization of immunization schedule by repeating and combining the different routes of mucosal immunization as a primary and boosting strategy can lead to the development of a new era of safe and effective mucosal vaccines [77].

7. Conclusion and Future Perspectives

Mucosal vaccination is a needle and medical waste free vaccine strategy that provides protective immunity against pathogenic bacteria and viruses in both mucosal and systemic compartments. However, the capabilities for the mucosal sites to induce humoral and cell mediated immune responses in each of the systemic compartment and the mucosal surfaces has not been entirely exploited as a result of the physical and chemical barriers that inhibit immune process activation. Vaccines developed from attenuated pathogens may not be always dependable, while inactivated pathogens mostly lack the ability to give total immune response. Other traditional subunit vaccines are prone to degradation and give low immunogenicity, thus requiring a more powerful delivery method. On the other hand, vaccines developed from the genetic material have the ability to give cell-mediated and humoral immune responses but for this purpose they require protecting and special delivery systems. Particle-mediated delivery systems can restrict degradation or premature neutralization of the antigens by utilizing the components of the immune systems and can deliver the vaccines to distinct tissues. They can be generated from various substances such as the polymers, lipids and metals and thus is an effective method to enhance mucosal vaccination by allowing the integration of adjuvant materials and defending the immunogenic materials during the specific delivery process.

Although mucosal vaccines are currently undergoing development for preventing mucosal infections and for treating allergic or autoimmune diseases, practical assays for assessing mucosal T reactivity in clinical and field settings are still rare and specific methods for predicting efficacy of mucosal vaccines in humans is still not available. In spite of significant advances in the development of improved mucosal vaccine delivery systems and novel mucosal adjuvants, the correlation of the safety and efficacy profiles established in animal models with the genetically diverse human subjects (exhibiting different intestinal flora, nutritional status and previous immunological experience) is yet to be elucidated. In fact, several mucosal vaccines including oral live cholera vaccine and rotavirus vaccine have been found to work less in the developing countries compared to the industrialized countries. These issues need to be addressed for development of mucosal vaccines. Therefore, a combination of appropriate mucosal vaccine strategies and new routes of mucosal immunization such as sublingual and ocular routes as well as novel approaches such as nanomatrix and plant based delivery systems may facilitate future mucosal vaccine development and targeting mucosal dendritic cells can overcome the two major hurdles of mucosal vaccines which are effectiveness and safety.

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