Review article

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Development of mucosal vaccine delivery: an overview on the mucosal vaccines and their adjuvants

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Currently, mucosal infectious diseases are still a very high global health burden, but there are few effective vaccines to prevent mucosal-borne diseases. The development of mucosal vaccines requires the selection of appropriate antigens, delivery system strategies, and adjuvants to increase vaccine efficacy but limited studies have been conducted. The aim of this review is to describe the mucosal immune system, as well as the potential for the development of vaccines and mucosal adjuvants, and their challenges. The study was conducted by applying inclusion criteria for the articles, and a review was conducted by two readers with the agreement. It was known that mucosal vaccination is a potential route to be applied in future preventive efforts through vaccination. However, limited studies have been conducted so far and limited mucosal vaccination has been approved. New technological approaches such as material development involving nano- and micro-patterning are important to intensively open and investigate the potential area of development to provide better vaccination methods.

Keywords: Mucosal-borne diseases, Mucosal vaccines, Delivery, Adjuvants

Introduction

Vaccines are one of the most important inventions in modern medicine and to date have provided protection from various disabilities in more than 750,000 children and the prevention of 3 million deaths each year [1]. Along with the increasing understanding of the mechanism of the mucosal immune response, one of the promising routes of vaccine administration is vaccination via mucosa because it can induce both a mucosal immune response and a systemic immune response [2-4]. Vaccination through the mucosa is expected to induce first-line immunity at the entry site of the pathogen, to prevent infection and its spread [5,6]. In addition, from several studies, it is known that the strongest immune response occurs in the mucosa exposed to the antigen and in the adjacent mucosa or in the mucosa that has specific connections [2,7].

Mucosal vaccine design requires appropriate antigen selection, delivery, and adjuvant system strategies to increase vaccine efficacy [6,8]. Adjuvants are components or agents added to vaccine formulas to help increase the stimulation of the immune system by increasing antigen presentation and/or providing co-stimulating/immunomodulatory signals as well as to deliver antigens into the immune system to produce a specific immune response [9-11]. However, only a few adjuvants have been approved for use in humans and most are used as part of parenterally administered vaccines.

The use of adjuvants for mucosal vaccines requires a review of the mechanisms and functions of these adjuvants on the mucosal immune system [12]. Therefore, an overview of the mucosal vaccine's mechanism, functions, and their adjuvants including state-of-the-art research in the area is needed.

Mucosal Immune System

The mucosa is the largest organ in humans with a surface area of 400 m^2 [13-15]. The mucosal surface requires protection because it is the first defense from pathogens that will invade the body [15]. The mucosal surface is composed of a thin and permeable layer to assist its physiological functions, including gas exchange (in the lungs), absorption of food (in the intestines), sensory activity (such as in the eyes, nose, mouth, and throat) as well as for reproduction (uterus and vagina). The presence of this permeable structure causes the mucosa to require an effective defense mechanism to prevent pathogen invasion [16]. Infectious diseases through the mucosa are still the highest infectious diseases in the world, including gastroenteritis, acute upper respiratory tract infections, pulmonary tuberculosis, influenza, human immunodeficiency virus (HIV) infection, and even infection by coronavirus. Apart from being the entry point for pathogens, the mucosa is also the entry point for non-pathogenic foreign antigens in the form of food proteins and commensal microbiota [17]. The mucosal immune system must be able to distinguish between non-pathogenic and pathogenic antigens so that an appropriate immune response is formed. Non-pathogenic antigens do not cause an immune response but form immune tolerance in the mucosal immune system [14].

The mucosal immune system is composed of an integrated network, lymphoid cells, non-lymphoid cells, and effector molecules including antibodies, chemokines, and cytokines that are responsible for aligning the innate immune response with the adaptive immune response when pathogens invade or the antigens administered during vaccination [16-18]. Fig. 1 shows the diagrammatic view of the mucosal immune system. All segments of the structure of the mucosal immune system are composed of anatomically and physiologically distinct induction and effector sites. Sites of induction include intestinal mucosa-associated follicles, such as Peyer's patches, isolated lymphoid follicles, and mesenteric lymph nodes where B cells and T cells undergo activation, clonal expansion, and differentiation into B and T effector cells. The B and T cells will migrate from the site of induction to the site of effector present in all parts of the mucosa. Anatomically and functionally, the mucosal immune system is divided into two main compartments, namely the induction site and the effector site [15,16].

The induction site area is composed of specific lymphoid tissue and is an area for antigen sampling which can then trigger the initiation of an antigen-specific immune response. Induction sites include gut-associated lymphoid tissue, nasopharynx-associated lymphoid tissue (NALT), and bronchus-associated lymphoid tissue. Overall, the site of induction in the mucosal immune system is called mucosal-associated lymphoid tissue (MALT) [19]. Meanwhile, the effector site is the area where antibodies and immune cells can perform specific functions after activation. Effector sites are present in all mucosal tissues in the form of lymphoid tissue scattered along the lamina or substance propria [19]. The effector site is also the site of antibody production and cell-mediated im-

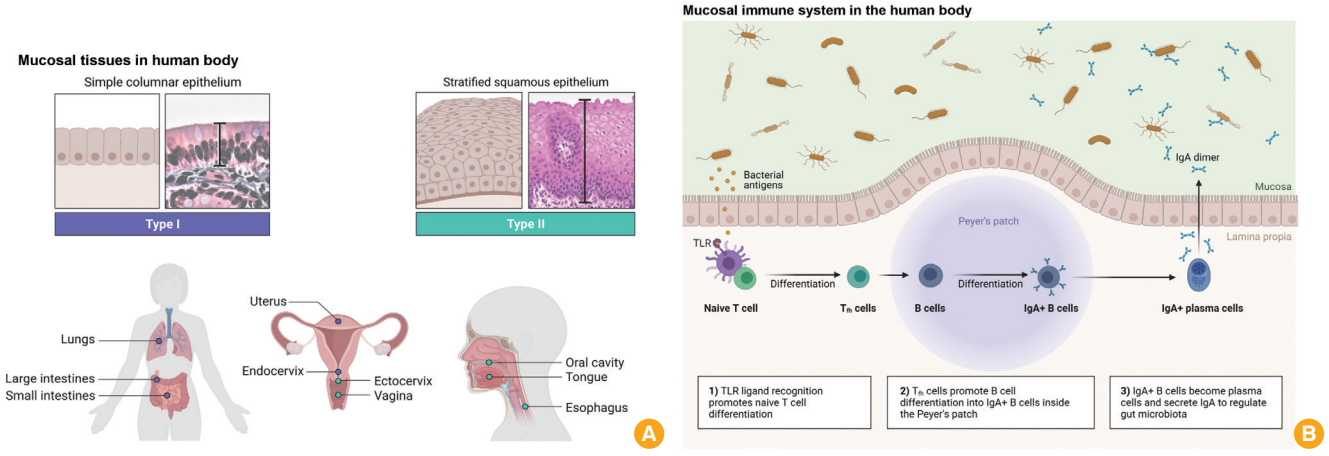

Fig. 1. Diagrammatic view of the mucosal immune system with mucosal tissue in human body (A) and overview of mucosal immune in human body (B). IgA, immunoglobulin A; TLR, Toll-like receptors.

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mune responses. The mucosal lymphoid cell population is estimated to be 80% composed of CD^{8+} and CD^{4+} T lymphocytes, which are the main effector cells on the mucosal surface [20].

There will be constant migration of antigen-induced immune cells from the induction site to the effector site. Mucosal-associated lymphoid tissue has a unique antigen sampling system, where antigens are represented by special cells called microfold cells (M cells) that are present in the epithelial layer of mucosal tissue that will help transport antigens to antigen presenting cells (APCs; dendritic cells, macrophages, B cells, and dendritic follicular cells) [21]. Sub-epithelial dendritic cells will also capture antigens at effector sites and migrate to local/regional lymph nodes which will then become active APCs that can stimulate T cells to become memory B cells or effector T cells. Naive B cells and T cells will enter MALT via high endothelial venules. Activated memory B cells and effector T cells after priming will migrate from MALT to the peripheral blood circulation and then extravasation will occur at the effector site [16,21].

Mucosal Vaccine

Vaccines are immunological preparations that are introduced into the body to induce an immune response against certain infections and/or diseases, with or without adjuvants. Vaccines may contain dead or attenuated pathogens, subunit antigens, viral vectors, nucleic acid-based RNA and DNA vaccines, and virus-like particles (VLPs) antigen [15,22,23]. Vaccination targets are healthy individuals, so the vaccine components must be safe, not cause non-specific immune responses and infections/diseases/adverse events. Ideally, vaccines should be able to trigger a sustained specific immune response against a pathogen and be able to induce specific neutralizing antibodies and protective T cells. In terms of distribution, ideally the vaccine is cheap and stable in storage, especially for providing vaccine doses in developing countries [15]. Vaccines can trigger several effector mechanisms of the immune system, through the activation of antibodies, CD^{8+} T cells and CD^{4+} T cells assisted by major histocompatibility complex (MHC) proteins. The viral antigen will bind to the MHC I protein which will then be presented by the APC to CD^{8+} cells and trigger cell-mediated immunity [24]. Bacterial/parasite antigen will bind to MHC II protein and be presented to CD^{4+} cells and trigger antibody-mediated immunity. Activation of CD^{4+} T cells by the vaccine will trigger the production of gamma interferon (IFN-γ), tumor necrosis factor (TNF)-α, TNF-β, interleukin (IL)-2, IL-3 which in turn helps the activation and differentiation of B cells [25].

The mucosal immune system is more accessible for induction of immune responses because the mucosal surface is the entry point for pathogens. In addition, vaccines in the mucosal area are expected to be able to induce first-line immunity at the site of entry of pathogens to prevent infection/disease and to produce a systemic immune response [26]. The mucosa as the first line of defense against various infections contains many dendritic cells that act as APCs that will present antigens to the immune system and then induce both mucosal and systemic immune responses to eliminate pathogens [2-4].

Up to this point, there are several mucosal vaccination routes that are currently being developed and require a strategy as an alternative method of vaccine administration. Research is needed on antigens that can cause specific immune responses, as well as appropriate delivery and adjuvant systems. Fig. 2 shows immunoglobulin A (IgA) immune response after vaccine administration from multiple routes. Several vaccination routes can produce an immune response in specific MALT areas. The strongest immune response will occur in the mucosa exposed to the antigen (site of administration) and in the adjacent mucosa or in the mucosa with specific connections [2,7]. Intranasal vaccination is effective in eliciting an immune response in the respiratory, gastrointestinal, and genital tracts. Oral vaccination is effective for eliciting an immune response in the intestines and breast glands. Vaccination via rectal is able to cause an immune response in the colon and rectal. While vaccination via intravaginal can cause an immune response in the genital tract [2,27].

The consideration for administering mucosal vaccines is

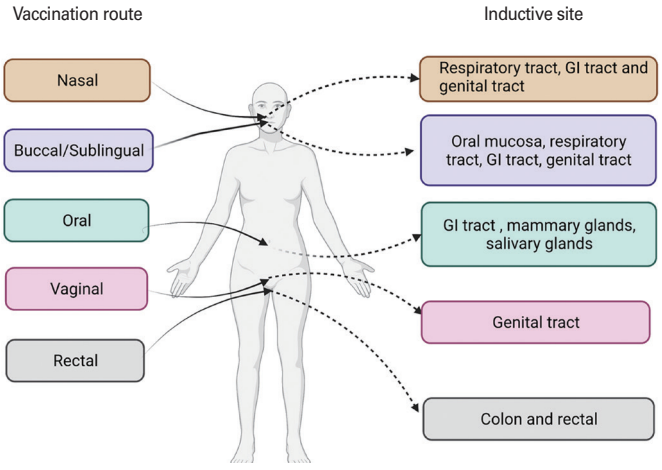

Fig. 2. Immunoglobulin A immune response after vaccine administration from multiple routes. GI, gastrointestinal.

that traditional vaccines via intramuscular injection induce only a small amount of mucosal immune response or no response at all, thereby reducing the effectiveness of preventing mucosal-borne infectious diseases [4,26]. Another reason for vaccination by the mucosal route is that most infections originate from mucosal surfaces, especially the nasal, oropharyngeal, respiratory, genitourinary, and gastrointestinal mucosa. The presence of infections originating or affecting the mucosa requires a new strategy in administering vaccines with the target of causing an immune response that can prevent infectious agents from attacking and colonizing the mucosal epithelium (non-invasive bacteria) or to prevent pathogens from penetrating and replicating in the mucosa (invasive viruses and bacteria), as well as preventing microbial toxins from binding to and affecting the epithelium and other target cells [2]. In general, the advantages of administering mucosal vaccines include increasing patient compliance due to the administration of vaccines without needles, reducing the risk of spreading infection from the use of syringes, reducing medical waste, and increasing the scope of vaccination programs, especially in developing countries because vaccines can be administered by non-medical personnel who have received training.

Some areas of the mucosa to be considered for vaccine administration are as follows. First, the oral mucosa can be considered as a mucosal vaccination route because it has advantages compared to other mucosal routes, including the oral mucous membrane has a relatively low enzyme activity, prevents the risk of antigen damage due to exposure to very low pH acids in the stomach, and is a safe route. Oral mucosa is also more comfortable for the patient than other routes and has a lower risk of complications in the central nervous system than vaccination via intranasal [4]. Administration of mucosal vaccines through the oral cavity can be through the sublingual and buccal mucosa. The anatomical structure and cell composition of the sublingual and buccal mucosa increase the effectiveness of vaccine administration because it is an area consisting of non-keratinized epithelium and has a thinner cell layer than another mucosa [4]. Vaccine antigens given through the sublingual and buccal mucosa will not enter the bloodstream directly but will be captured by dendritic cells, especially Langerhans cells which will then present to T cells. Dendritic cells that carry antigens will migrate to the sublingual and buccal lymph nodes, which in turn will activate CD^{4+} naive T cells and CD^{8+} cells. During activation, T and B cells enter the circulation and then differentiate into memory cells or effector cells. Activation of CD^{4+} T cells will induce helper T cells (Th cells), while CD^{8+} T cells will induce a cytotoxic T lymphocyte response [3,4]. There is a distinct subset of dendritic cell populations in the buccal and sublingual mucosa that act as APCs. In the buccal mucosa there are many populations of Langerhans cells, while in the sublingual mucosa there are many interstitial dendritic cells [3].

Research by Appledorn et al. [28] concluded that the administration of adenovirus serotype 5-based vaccine dripped onto the sublingual mucosa in adult male C57BL/6 mice could induce a specific immune response to HIV antigen with the formation of Gag-specific cytotoxic T-lymphocytes (CTL). Cuburu et al. [29] concluded that vaccination using human papillomavirus (HPV)16 L1 VLPs given alone or with cholera toxin adjuvant which was applied topically sublingually to female BALB/c and C57BL/6 mice could effectively induce a genital immune response, particularly for protection against human papillomavirus pseudo virions by inducing virus-neutralizing responses in genital secretions. Raghavan et al. [30] found that sublingual vaccination of female C57BL/6 mice using *Helicobacter pylori* lysate and cholera toxin adjuvants resulted in IgA and IgG antibody responses in the stomach and intestines, increased production of IFN-γ and IL-17 production by the spleen and intestine mesenteric lymph node T cells, and increased expression of IFN-γ and IL-17 genes in the stomach compared to mice that were not given.

Fig. 3 shows mechanism of immune response induction after sublingual or buccal vaccination. It is shown that antigen will be captured by Langerhans cells and myeloid dendritic cells then will migrate to draining lymph nodes and interact with CD^{4+} and CD^{8+} T cells which will then induce an adaptive immune response. Furthermore, there will be migration to effector sites and interconnected sites.

The nasal mucosa is a highly immune-competent area so small doses of antigen can elicit a protective response. Vaccination through the nasal mucosa will provide both local and systemic immune responses [31]. Intranasal vaccination will induce an immune response in the mucosa of the upper airway as well as in other effector areas, such as the lungs, intestines, and genitals [2,31]. The advantage of intranasal vaccine administration is the presence of microvilli in the epithelium which can increase antigen uptake and can induce mucosal and systemic immune responses. The challenge of administering intranasal vaccines is the presence of a natural defense barrier that must be passed by mucosal antigens in order to penetrate the mucosa and reach target cells/tissues. Substances that enter the nostrils will be blocked by hair and ke-

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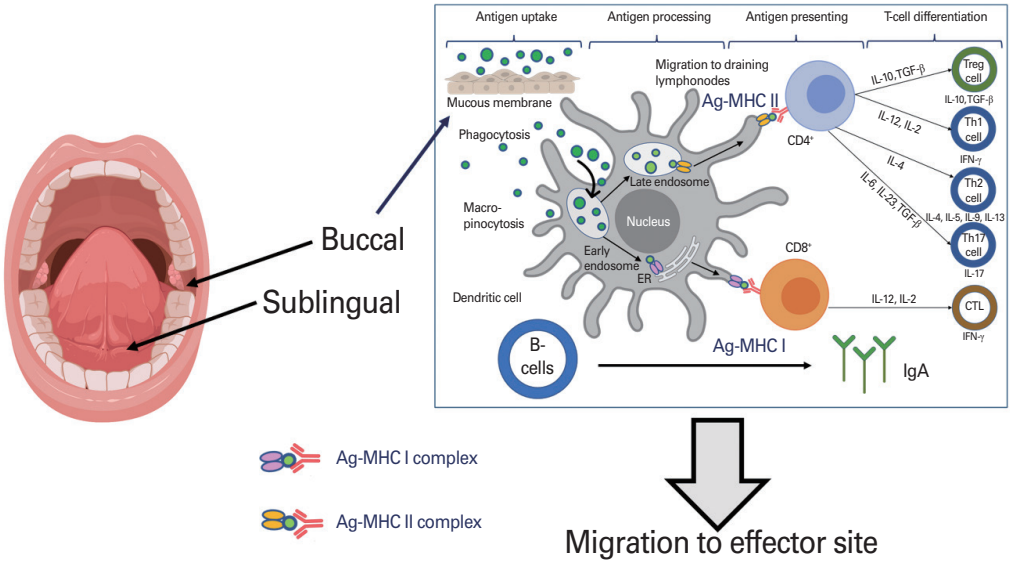

Fig. 3. Mechanism of immune response induction after sublingual or buccal vaccination. MHC, major histocompatibility complex; IgA, immunoglobulin A; CTL, cytotoxic T-lymphocytes; IL, interleukin; TGF-β, transforming growth factor-β; IFN-γ, gamma interferon; Th cells, helper T cells.

ratinized stratified squamous epithelia in the mucosal lining of the nostrils (nostrils). Physical barriers such as cilia and mucus in the upper and lower respiratory tract will naturally direct foreign substances/agents to the oropharynx which will then be swallowed and degraded by gastric acid [32].

Arevalo et al. [33] performing intranasal vaccination with adenoviral vectors of *Streptococcus pneumoniae* strain D39 in BALB/c female mice resulted in an adequate immunoglobulin G antibody response. Ainai et al. [34] gave vaccines from inactivated influenza A (H5N1) virus using intranasal spray to human volunteers and it was adequate to induce a nasal IgA antibody response but did not produce an IgG antibody response. Research conducted by Hassan et al. [35] demonstrated that administration of a single dose of the adenoviral vaccine ChAd-SARS-CoV-2-S can induce neutralizing antibodies, increase systemic and mucosal IgA as well as T-cell responses, and almost completely prevent SARS-CoV-2 infection of the upper and lower respiratory tract in adult mice during the challenge test. Fig. 4 shows mechanism of immune response induction after intranasal vaccination. Antigens will be captured by dendritic cells and other APCs present in epithelial cells and follicle associated epithelium then will migrate to NALT and interact with $CD4^+$ and $CD8^+$ T cells which will then induce an immune response.

Oral vaccine is a mucosal vaccination that was initially developed. Several licensed vaccines such as the oral polio vaccine (OPV) have been widely used in immunization programs around the world. However, around 2000 there were outbreaks of polio in Haiti, the Philippines, Egypt, and Dominica caused by reversion of the attenuated virus to infectious wild type strains originating from OPV, causing infection in immunocompromised individuals [36]. Based on this, safety considerations for the development of oral vaccines are very important. In addition, a better vaccine delivery system is needed because oral administration will cross barriers, such as low pH and acid in the stomach [37]. The advantage of administering oral vaccines is that the intestine is home to commensal microbiota, so that the purification process for the given antigen will be simpler because it does not require high purity [36].

Serradell et al. [38] investigated a chimeric VLP containing a variant specific surface protein from the influenza virus hemagglutinin (HA) that has been administered orally. They found that the method can produce a strong immune response against influenza infection in mice. Research conducted by Barackman et al. [39] using influenza HA in combination with mutant *Escherichia coli* heat-labile enterotoxins K63 (LT-K63) and R72 (LT-R72) as an adjuvant showed an increase in potent serum antibodies, HA inhibition titers, and HA-specific IgA in saliva and nasal secretions after oral administration. Fig. 5 shows main principles of immune response induction after oral vaccination. Peyer's patches are surrounded by a collection of specialized cells called M cells which interact with the antigen and are transported to the APC, wherein in the next cascade will be the induction of an immune response.

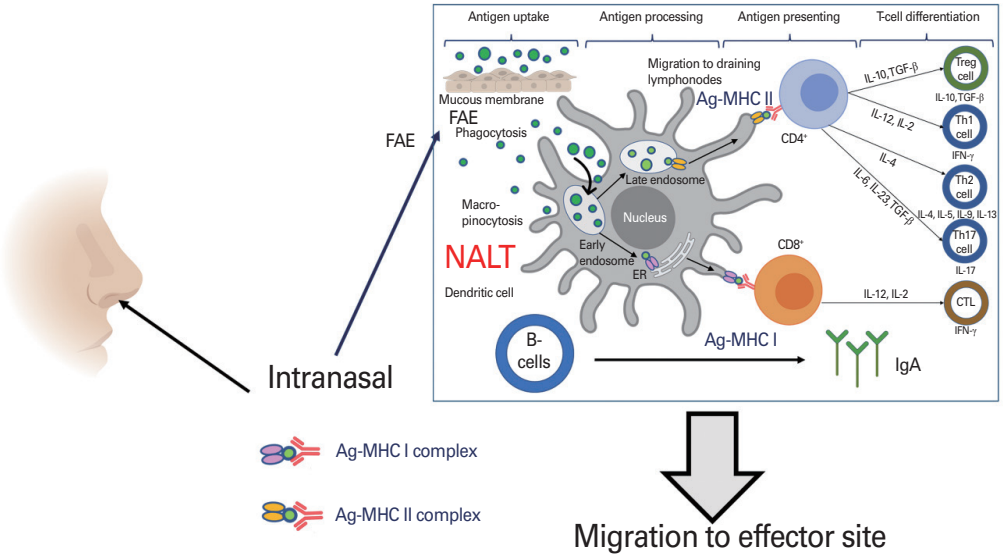

Fig. 4. Mechanism of immune response induction after intranasal vaccination. MHC, major histocompatibility complex; IgA, immunoglobulin A; CTL, cytotoxic T-lymphocytes; IL, interleukin; TGF-β, transforming growth factor-β; IFN-γ, gamma interferon; NALT, nasopharynx-associated lymphoid tissue; FAE, follicle associated epithelium; Th cells, helper T cells.

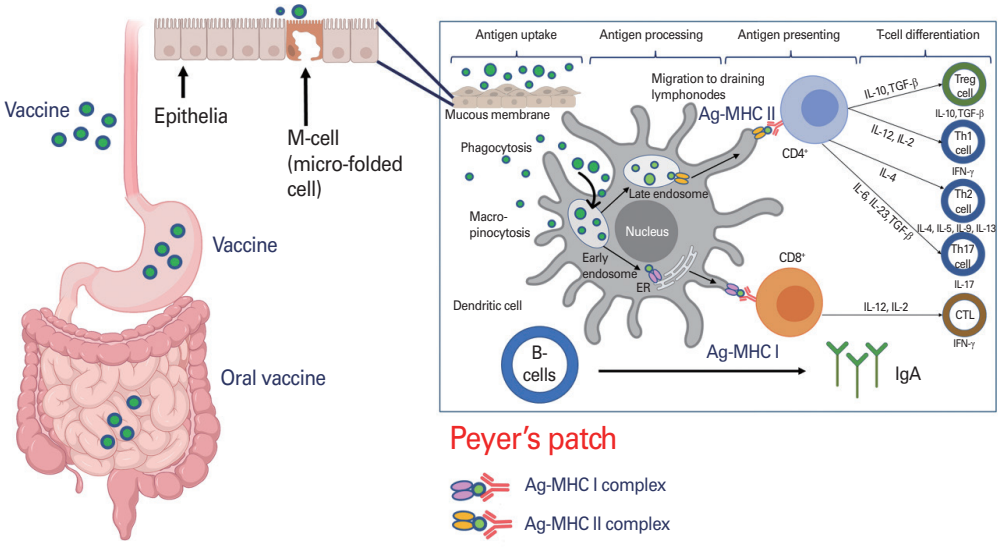

Fig. 5. Main principles of immune response induction after oral vaccination. MHC, major histocompatibility complex; IgA, immunoglobulin A; CTL, cytotoxic T-lymphocytes; IL, interleukin; TGF-β, transforming growth factor-β; IFN-γ, gamma interferon; Th cells, helper T cells.

Aside from the oral mucosal vaccination through sublingual and buccal, intranasal, and oral mucosal, vaginal mucosa is also categorized into mucosal system. The vaginal mucosa is a component of the mucosal immune system, although it has several different features compared to other mucosal sites. In general, the male and female genital tracts have only a few inductive mucosa sites and thus produce only a low humoral and cellular immune response in the presence of infection [40]. However, several studies have shown that intravaginal local administration of vaccines can induce an adequate immune response, both humoral and T cell-mediated immune responses, so that intravaginal administration of vaccines can be considered as a route of vaccine administration, especially for the prevention of sexually transmitted diseases [8,27].

Several studies have developed vaccine candidates for the prevention of HIV and HPV by administering intravaginal vaccines [37,41]. Johansson et al. [42] conducted research to investigate the comparison of cholera toxin B subunit to human volunteers vaccinated with mucosal antigen model,

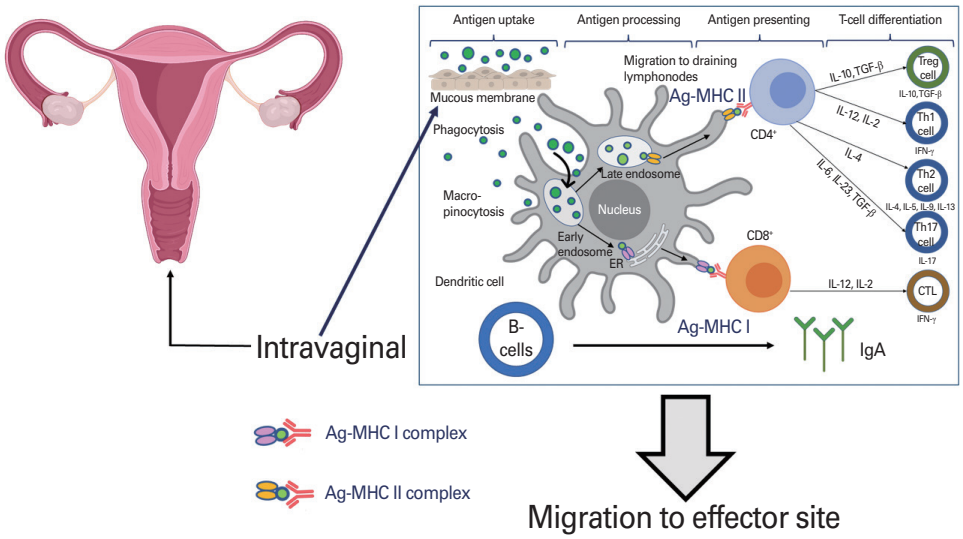

Fig. 6. General principles of immune response induction after intravaginal vaccination. MHC, major histocompatibility complex; IgA, immunoglobulin A; CTL, cytotoxic T-lymphocytes; IL, interleukin; TGF-β, transforming growth factor-β; IFN-γ, gamma interferon; Th cells, helper T cells.

both intranasally and intravaginally, to see the specific immune response of IgA and IgA both systemic and in vaginal and cervical secretions in the administration of the vaccine with these two routes. In this study, it was shown that the administration of intranasal and intravaginal vaccines could significantly increase the specific systemic immune response of IgA and IgG, while the specific immune response of IgA and IgG in cervical secretions was only produced in the group given the intravaginal vaccine. Intranasal administration of vaccines can increase the specific IgA response in vaginal secretions. The results of this study indicate that the combination of intranasal and intravaginal vaccines can induce an antibody response in vaginal and cervical secretions.

Hopkins et al. [43] conducted a phase 2 clinical trial of an intravaginal vaccine for the prevention of urinary tract infection (UTI) by administering an intravaginal suppository containing heat-killed bacteria from 10 human uropathogenic strains. In this study, there was an increase in the specific immune response of IgA and IgG in urinary and vaginal secretions in the vaccinated group but there was no statistically significant difference. In the analysis of the recurrence rate of UTI, there was a significant difference between the group that was given the vaccine and the placebo group which showed that the administration of the vaccine could reduce the recurrence rate of UTI caused by *E. coli*. Fig. 6 shows general principles of immune response induction after intravaginal vaccination. The vaccine antigen will be captured by dendritic cells and other APCs which will then interact with CD^{4+} and CD^{8+} cells and induce an adaptive immune response.

In view of the recent developments on mucosal vaccination, Table 1 shows pros and cons on mucosal administration routes for considerations. Furthermore, in recent decades, the development of vaccine technology platforms has begun to change from conventional platforms such as whole killed or attenuated viruses to subunit vaccines, VLP, RNA, and DNA-based vaccines. The platform has only been used as a parenteral vaccine and there is no licensed mucosal vaccine yet [6,8,44]. The existing and licensed mucosal vaccines are live attenuated and whole-killed virus vaccines, as shown in Table 2.

Adjuvant for Mucosal Vaccination

Adjuvants are derived from the word "adjuvare" which means "to help", and are components or agents added to vaccine formulas to help increase stimulation of the immune system by increasing antigen presentation and/or providing co-stimulatory/immunomodulatory signals as well as to deliver antigens into the immune system so that a response generated specific immunity [9-11]. The purpose of adding adjuvants to the vaccine formula is to increase the immune response and maintain the sustainability of the immune response in the body. The presence of adjuvants can reduce the frequency of vaccine boosters and can reduce the amount of antigen needed in a vaccine formula. In addition, adjuvants can also be used to increase antibody responses in mucous membranes, which are the body's first line of defense [10,11].

Table 1. Pros and cons on mucosal administration routes

Route	Pros	Cons
Oral mucosa (buccal/sublingual)	- Composed of non-keratinized stratified squamous epithelium	- Risk of antigen ingestion
	- Has relatively low enzyme activity	- Need to consider the dose of antigen as well as the right delivery system
	- Has a lower risk of complications in the central nervous system	
Intranasal	- Composed of immunocompetent cells	- Composed of keratinized stratified squamous epithelium
	- Contains microvilli-equipped epithelial cells that can increase antigen uptake	- Risk of complications in the central nervous system
	- May induce an immune response in the lung, intestinal, and genital areas	- Must pass through natural barriers such as cilia and mucous
Oral	- Easiness in application	- Has a natural barrier in the form of low pH and acid in the intestine
	- There is gut microbiota colonization which can help induce immune response	- Risk of antigen damage due to exposure to acid in the intestine
Intravaginal	- Can induce a mucosal immune response in the genital area	- The method of administering the vaccine in the male genital area needs special consideration
		- Hormone levels can affect the immune response in the female genital

There are two types of adjuvants, namely immunostimulants and "vehicles" that can carry vaccines to the immune system [45,46]. Adjuvants can be in the form of molecules, compounds, or macromolecular complexes that can increase the potency, quality, and duration of specific immune responses to certain antigens with minimal toxicity. Although in general adjuvants can be classified as immune modulators or vehicles for delivery, this classification is currently starting to get confusing because some components are known to have the ability to perform these two functions [46]. Administration of vaccines without adjuvants results in repeated injection of vaccines to achieve a therapeutic effect, while injection of vaccines with adjuvants can increase the interaction between antigens and/or adjuvants with immune cells and can further increase the potential for formation of immune cells [10]. The repeated administration of booster vaccines will certainly be a problem in developing countries because access to health care facilities is still limited.

Adjuvants in general can increase the response of B and T cells which cooperate with the innate immune system to cause an immune response to specific antigens and are able to increase the adaptive response to vaccination, as measured by antibody titers or the ability to prevent infection. In addition, adjuvants have another role, namely, to direct the type of adaptive immune response produced so that the most effective immune response is formed for each antigen. The purpose of using adjuvants in vaccination is to increase the immunogenicity of the antigen, thus, reducing the amount of antigen given during vaccination and the dose of injection. Adjuvants also increase the response to vaccination in the population, so as to increase antibody titers and/or the fraction of subjects protected by immunization; thus increasing seroconversion rates in low-response populations (e.g., in infants and the elderly, who have certain diseases, or are undergoing therapeutic interventions) as well as increasing vaccine efficacy in newborns, the elderly, and people with low immune systems and functioning as an antigen delivery system for increased antigen uptake [10,47,48].

Although adjuvants are an important component in vaccine formulas, only a few adjuvants are approved for use in humans [15]. Some considerations in the use of adjuvants include the antigen to be administered, the species to be vaccinated, the route of administration, and possible side effects. Ideally, the criteria that must be possessed by adjuvants are non-toxic, can stimulate humoral and cellular immunity, can induce immunological memory, non-mutagenic, non-carci-

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Table 2. Licensed mucosal vaccines [6,8,40]

Pathogen	Platform	Trade name	Route	Formulation
Poliovirus	Live attenuated	Biopolio (bOPV), mOPV, and tOPV	Oral	Liquid
Rotavirus	Live attenuated/Live reassortant	Rotateq, Rotarix	Oral	Liquid
Vibrio cholerae	Inactivated	Dukoral, Euvichol, Shanchol	Oral	Liquid
Vibrio cholerae	Live attenuated	Vaxchora	Oral	Liquid
Salmonella thyphimurium	Live attenuated	Vivotif	Oral	Enteric coated capsule
Influenza A and B viruses	Live attenuated	FluMist, Fluenz Tetra	Nasal	Spray

nogenic, non-teratogenic, non-pyrogenic, stable on storage (in terms of time, temperature, and pH), biodegradable, low production cost, non-antigenic/no immune response to the adjuvant, and able to increase the appropriate immune response [47,49].

The benefits conferred by the addition of adjuvants to vaccines should not be accompanied by adverse effects. Therefore, it is necessary to select an adjuvant from a safe and non-toxic substance. Several studies have shown that some adjuvants are potent but have a high level of toxicity, including Freund's Complete Adjuvant [50]. One of the challenges faced for adjuvant development is to obtain potent adjuvants with low toxicity and even non-toxicity. This difficulty is a major problem that causes only a few adjuvants to be permitted for use in humans [48,51].

The adjuvant mechanism in eliciting the immune response is not completely clear, but there are several mechanisms that are thought to be the effect of the adjuvant in enhancing the mucosal immune response. Local inflammation triggered by adjuvant-antigen combinations can elicit an adaptive immune response by recruiting APC [8]. The first stage is the presence of antigen uptake on the mucosal surface, small adjuvant particles can passively diffuse and cross the barrier through the intercellular space mediated by neonatal Fc receptor or claudin 4 [52]. Bacterial-derived adjuvant will be captured by M cells or by intraepithelial dendritic cells. Antigens that successfully cross the mucosal barrier will be captured by dendritic cells residing in the mucosa and will then migrate to draining lymph nodes [53]. Although in general the increased immune response by adjuvants is due to the presence of pro-inflammatory mechanisms, non-inflammatory pathways are currently being considered to enhance vaccine safety [54]. Some adjuvants, such as polysaccharide-based adjuvants, are known to have non-inflammatory mechanisms that have a higher level of safety compared to pro-inflammatory pathways [55]. The following is description on some mucosal vaccination related adjuvants.

Bacterial enterotoxin

Bacterial enterotoxins that are often used as adjuvants in several mucosal vaccine studies are cholera toxin produced by several *Vibrio cholerae* strains, LT produced by several *E. coli* strains, as well as cholera toxin and LT subunits [56,57]. The addition of enterotoxin as a mucosal adjuvant can increase the permeability of the mucosal barrier so that it can increase recruitment and local activation of APCs and can induce specific IgA antibodies and memory immune responses [58]. However, the addition of enterotoxins is often associated with some undesirable side effects, such as Bell's palsy paralysis in some individuals after intranasal administration and some occurrence of diarrhoea after oral administration [27]. The enterotoxin subunit or commonly known as the detoxified mutant is considered safer than LT [59].

Barackman et al. [39] administered oral influenza HA vaccination in combination with mutant *E. coli* LT-K63 and R72 (LT-R72) and showed an increase in IgG systemic immune response and specific mucosal immune response IgA in salivary and nasal secretion. In the previous study, Sjökvist Ottsjö et al. [60] combined double mutant *E. coli* heat-labile toxin (dmLT) with *H. pylori* lysate antigens and administered them sublingually. They found an increase in the specific immune response of IgA in the stomach, increased proliferation in mesenteric lymph nodes and spleen cells, upregulation of the expression of the cytokine gene IFN-γ, IL-17, TNF, and CD^{4+} genes.

In general, the mechanism of increasing the immune response with a combination of LT, cholera toxin, or dmLT due to the mucosal binding properties of these enterotoxins will then increase the ability of antigens to bind to the mucosa (mucosal binding). Antigens will bind to neuraminic acid-rich glycoproteins while enterotoxins will bind to ligands in the mucosa [39].

TLR agonist

Toll-like receptors (TLR) are a sub-category of pattern recog-

nition receptors that can bind to ligands on pathogenic microorganisms or are called pathogen-associated molecular patterns [61]. TLRs can mediate intracellular signaling pathways that can trigger the production of pro-inflammatory cytokines and up-regulation of MHC molecules. TLRs can also induce Th1 and Th2 cell responses, CD^{4+}, and CD^{8+} cell development, and amplify B and T cell responses [62]. The B and T lymphocytes expressing TLR and TLR agonists can directly modulate the function of these cells [63,64]. Velasquez et al. [65] administered the vaccine via intranasal use of Norwalk virus VLP with TLR-7 agonist as a co-deliver. They showed the induction of systemic IgG-specific immune responses and specific mucosal immune responses of IgA in the gastrointestinal, respiratory, and reproductive tracts in mice.

Cytokines

Cytokines are signaling proteins released by cells and have an important role in the activation and regulation of innate and adaptive immune responses, as well as humoral and cellular immune responses [66]. Cytokine molecules are considered as one of the potential adjuvants because they can enhance the immune response and vaccine efficacy [67]. Cytokines play a role in the differentiation of helper T cells into Th1 and Th2 cells depending on the cytokine environment [68]. Upon exposure to antigen, IL-2 will be produced by naive CD^{4+} T cells and act as a growth factor [68]. IFN-γ together with IL-12 will trigger differentiation into Th1 cells [64]. IL-4 and IL-2 play a role in differentiation into Th2 cells [69,70].

Parenteral administration of cytokines has safety issues because of their toxicity; however, when administered through the mucosa such as via the intranasal route, the toxicity is low [68]. Kayamuro et al. [71] conducted an examination on the mucosal and systemic immune response against influenza virus induced by IL-1 family cytokines which showed that IL-1α, IL-1β, IL-18, and IL-33 were effective mucosal adjuvants to induce secretory IgA and CTL immune responses on administration via intranasal.

Nanoparticles

Both adjuvants and nanoparticle-based delivery systems have the potential to be developed in mucosal vaccines because they have favorable characteristics, such as being surface modified, having controlled release capability, and increasing the stability of the antigen being carried [72]. Nanoparticles can come from organic or inorganic materials. Several studies of mucosal vaccines have used nanoparticles derived from calcium phosphate (CP), chitosan, poly (L-lactic acid) or PLA, and poly (Lactic-co-Glicolyc acid) or PLGA. Study by He et al. [73] vaccinated herpes simplex virus type 2 antigens via intranasal and intravaginal using CP adjuvants in which they showed the induction of specific IgA and IgG immune responses and the formation of neutralizing antibodies in the serum of experimental mice. In the other study, Thomas et al. [74] used several nanoparticulate formulations of PLA or PLGA with hepatitis B surface antigen and administered the vaccine via aerosol to the lungs in experimental mice. Evaluation of the mucosal immune response showed a significant increase in the level of specific IgA in salivary secretion, vaginal secretion, and bronchoalveolar lavage in addition to an increase in IL-2 and IFN-γ levels in the spleen. In a study conducted by Prego et al. [75], it was observed that an intranasal vaccine with a combination of chitosan-based nanoparticles and recombinant hepatitis B surface antigen showed the formation of an immune response at a seroprotective level as indicated by an increase in specific IgG. In this study, the mucosal immune response was not evaluated. Although the mechanism of the increasing immune response by nanoparticles is not yet fully known, it is possible because nanoparticles can reduce barriers to cellular uptake and increase endocytosis by dendritic cells [73,74].

Challenges on the Development of Mucosal Vaccination

The development of mucosal vaccines has several challenges, including that the delivery of antigens to target cells or tissues is less consistent than vaccination via intramuscular or subcutaneous injection because they must pass through the mucosal barrier which is the body's natural defense mechanism [76]. Antigens from mucosal vaccines must be able to survive through barriers such as mucous flow, gastric acid, mucosal antibodies, and epithelium-derived antimicrobial peptides (such as defensins, cathelicidin, and histatins) which naturally destroy foreign agents present on the mucosal surface [77]. Therefore, mucosal vaccines require higher doses of antigen as well as appropriate delivery technology for both administration and micro delivery methods for antigen [78].

Another challenge in the development of mucosal vaccines is related to the standard limits of the immunological effects produced after administration of the vaccine. Traditionally, the immunological effect of vaccination would be calculated

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based on the antibody titer produced. In administering mucosal vaccines, it is necessary to review the standard of immunological examination tests produced after administration of the vaccine. Examination of the mucosal immune response will be limited due to limited access to mucosal sampling. Some sampling of the mucosa, such as in lung or intestinal tissue will require invasive measures so that it is necessary to review the standard of mucosal immune response that can be applied to patients [8,32].

In addition, in the development of mucosal vaccines using experimental animal models, it becomes a separate obstacle because of the fundamental differences regarding the anatomy and types of human mucosal epithelium and experimental animals in general. So that there will be a gap in extrapolation of research results from experimental animal models to humans [79]. Another challenge is the vaccine platform use because some vaccines derived from intact organisms can revert to infectious pathogens in some individuals, especially in immunocompromised individuals [32].

Conclusion

In conclusion, mucosal vaccination is considered promising routes for vaccine administration because it can induce both a mucosal immune response and a systemic immune response. This is due to the capability of mucosa to induce first-line immunity at the entry site of the pathogen, to prevent infection and its spread. However, limited studies have been conducted so far due to pros and cons in the mucosal vaccination, thus it limits number of licensed and approved mucosal vaccination. In view of this, exploration, and much more intensive research on the development of mucosal vaccine adjuvants are needed to response the challenges on the certain barriers for mucosal vaccination. The use of recent advanced technologies such as nanoparticles development, micro, and nanopatterning may be useful in the fabrication of barriers free mucosal vaccine adjuvants. Standardized protocols for immunogenicity testing related to mucosal vaccination are also an open area to study.

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