Mucosal Delivery of Vaccines

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Certification Statement

This is to certify that this project titled ‘Mucosal Delivery of Vaccines’ submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Ms. Zara Sheikh, Department of Pharmacy, BRAC University and that appropriate credit is given where I have used the language, ideas or writings of another.

Signed,

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Countersigned by the Supervisor,

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Dedicated to my Parents, who have sacrificed their earthly happiness in fulfilling my ones and also to my beloved siblings and friends.

Abstract

Vaccines are capable of inducing cellular and humoral immune responses which could provide prophylactic and therapeutic responses against various diseases such as those that are infectious in nature and other that are malignant in nature such as cancer. These responses can also be stimulated systemically and at the mucosal surfaces by activating the mucosal immune process. However most licensed vaccines are administered parenterally and fail to elicit protective mucosal immunity. Immunization by mucosal routes may be more effective at inducing protective immunity against mucosal pathogens at the site of entry. Different challenges are associated with different varieties 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 overcome these challenges and thereby enhance the mucosal vaccination. The usage of particulate delivery is an effective method that can be taken up to enhance mucosal vaccination. This is done by protecting the immunogenic material during the delivery, facilitating specific target oriented delivery system, and by allowing incorporation of various adjuvant materials. Efforts are focused on efficient delivery of vaccine antigens to mucosal sites that facilitate uptake by local antigen-presenting cells to generate protective mucosal immune response. Future issues regarding mucosal vaccine development have also been pointed out 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 antigen delivery systems along with novel mucosal adjuvants.

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LIST OF ABBREVIATIONS

C- Complement System

MBL-Mannose Binding Lectin

PRRs- Pattern Recognition Receptors

TLRs- Toll-Like Receptors

NLRs- NOD like Receptors

Pamps- Pathogen Associated Microbial Patterns

DNA-Deoxyribonucleic Acid

RNA- Ribonucleic Acid.

mRNA-Messenger RNA.

NKs- Natural Killer Cells

IgD- Immunoglobulin D

IgA- Immunoglobulin A

IgE- Immunoglobulin E

IgM- Immunoglobulin M

MHC – Major Histocompatibility Complex

MALT- Mucus Associated Lymphoid Tissue

BALT – Bronchus Associated Lymphoid Tissue.

GALT-Gut Associated Lymphoid Tissue.

pIgR-Polymeric Immunoglobulin Receptor

sIgA-Secretory Immunoglobulin A

DCs-Dendritic Cells.

M Cells- Microfold Cells.

ADCC- Antibody Dependent Cell Mediated Cytotoxicity

CLTs- Cytotoxic T Lymphocytes

IFns – Interferons.

FCrn- Neonatal FC Receptors

HIV- Human Immunodeficiency Virus

FAE- Follicle Associated Epithelium

MIP3α - Macrophage-Inflammatory Protein 3α

CC- Chemokine Receptor

ELISA- Enzyme Linked Immunosorbent Assay

APCs- Antigen Presenting Cells

CVM- Cervicovaginal Mucus

CD- Characterized DC Receptor

DCSIGN- DCSpecific Intercellular Adhesion Molecule 3-Grabbing Non Integrin.

PLGA–Poly Lactic Co-Glycolic Acid.

NLR- NOD likeReceptor.

ISCOM- Immune Stimulating Complexes

CpG- Cytosine-Guanine Phosphodiester Bond

CT- Cholera Enterotoxin

LT-Heat Labile Enterotoxin

HPV- Human Papilloma Virus

Hbs Ag- Hepatitis B Surface Antigen

IRIVIS-Immunopotentiating Reconstituted Influenza Virosomes

BCV-Bacterial Ghosts Vector

CTA1-DD-Cholera Toxin Subunit A1–Staphylococcus Aureusprotein A D-Fragment Dimer

CTL-Cytotoxic T lymphocyte

DDA-Dimethyl Dioctadecyl Ammonium

iNKT-Invariant Natural Killer T Cells

MDP-Muramyl Dipeptide

MPL-Monophosphoryl Lipid A

ND-Not Determined

T_h-T Helper

UEA1-Ulex Europaeus Agglutinin 1

VLP-Virus like Particle

LPS-Lipopolysaccharide.

Chapter-1

INTRODUCTION

1. INTRODUCTION

Mucosal surfaces are enormous surface areas that are a common site of entry for pathogenic microorganisms (Neutra, 2006). 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. This clearly indicates the importance of local mucosal immune responses for protection against disease, as for example, mucosal antibodies against *Vibrio cholerae* bacteria and cholera toxin is associated with resistance to cholera (Levine, 2000). Mucosal immunization through oral, nasal, rectal or vaginal routes can effectively induce mucosal immune responses rather than the vaccines that are injected. Therefore the vaccines that are administered onto the mucosal surfaces have proved to be more efficient in producing mucosal immune responses than injected vaccines (Neutra, 2006; Levine, 2000).

Vaccines are biological preparations that enhance immunity against disease and either prevent (prophylactic vaccines) or treat disease (therapeutic vaccines) (Delany et al, 2014). The word 'vaccine' originates from the Latin word *Variolae vaccinae* (cowpox), which Edward Jenner demonstrated in 1798 could prevent smallpox in humans. The research based on clinical vaccine is focused primarily on injection of antigens. The route of administration of vaccines is mostly either intramuscular or subcutaneous. An injection delivers a known quantity of antigen into the body that result in the generation of specific antibodies and lymphoid cells which can be readily measured in blood samples. On the contrary, mucosal immunity and development of mucosal vaccines faces many obstacles owing to the fact that administration of mucosal vaccines and measurement of mucosal immune responses are rather more complicated. The dose of mucosal vaccine that actually enters the body cannot be accurately measured because antibodies in mucosal secretions are difficult to capture and quantitate, and recovery and functional testing of mucosal T cells is labour intensive and technically challenging.

When compared with synthetic vaccines in terms of production and also regulatory purposes the mucosal vaccines have more advantages. For instance, vaccines that are administered orally do not require extensive purification from the bacterial by-products this is because the intestine is already populated by means of microorganism, whereas the same vaccine components when

injected parenterally is needed to be purified as it might have unacceptable endotoxin levels. Furthermore, mucosal vaccines are more realistic for mass vaccination as they do not contain the danger of spreading blood-borne infections that can be contaminated injection needles. The mucosal vaccines also has the advantages of the convenience of administration, better compliance and they can be possibly delivered by personnel without any sort of clinical coaching particularly for preventing the pandemic spread of infections such as influenza. (Lycke, 2012)

Quite a few numbers of mucosal vaccines have been approved for human use in the United States or elsewhere (Table 1). These include oral vaccines against poliovirus, *Salmonella typhi*, *V. cholerae* and rotavirus, and a nasal vaccine against influenza virus. Research on mucosal vaccines is advancing at an increasing rate. The present review gives an overview about mucosal immunity and key biological and technical aspects of design and development of mucosal vaccine.

Vaccine	**Trade name**	**Composition**	**Dosage and formulation**	**Mechanism of protection**	**Efficacy**
Influenza type A and B viruses	FluMist	Live viral reassortant with trivalent mix of H1, H3 and B strains of haemagglutinin and neuraminidase genes in an attenuated donor strain	Intranasal in young children, 2 doses	Haemagglutinin- and neuraminidase-specific mucosal IgA and systemic IgG responses; possibly a role for cell-mediated immunity; heterotypic protection effective in children	>85% in children, variable

Tabel 1 (Continued)

Virus (swine flu)	NASOVAC	Monovalent live attenuated vaccine	Intranasal spray, 1–2 doses	Haemagglutinin- and neuraminidase‑specific mucosal IgA and serum IgG antibodies; possibly cell-mediated immunity	Unavailable
Rotavirus	RotaTeq ; Rotarix	Monovalent, live attenuated human rotavirus and multivalent animal–human reassortant rotavirus	Oral, 3 doses	Mucosal IgA and systemic neutralizing IgG antibodies specific for homotypic or heterotypic VP4 and VP7 antigens	>70–90% against severe dise
Poliovirus	Many	Trivalent, bivalent and monovalent vaccines	Oral, 3 doses	Mucosal IgA and systemic IgG neutralizing antibodies	>90% in most of the world
Salmonella Typhi	Vivotif ; Ty21A	Live attenuated S. Typhi (Ty21A)	Oral, 3–4 doses of Ty21A	Mucosal IgA and systemic IgG antibody responses and CTL responses	Variable, but >50%

Table 1 (Continued)

Vibrio cholerae	Orochol	Live recombinant vaccine lacking CTA (CVD 103HgR)	Oral, single dose	Vibriocidal antibodies (possibly not the main effector mechanism but correlate well with protection)	Poor effect in a field trial
Cholera	Dukoral ; Shanchol	Whole killed Vibrio cholerae O1 classical and El Tor biotypes with (Dukoral) or without (Shanchol) CTB	Oral, 2–3 doses	Gut antitoxin- and CTB-specific IgA and antibacterial and LPS-specific antibodies	Strong herd protection; >85% short term; >60% 3–5 years

Table 1: Licensed Mucosal Vaccines (adapted from Lycke, 2012)

1.1. Components of The Immune System

Immunity means protection against diseases. The immune system is comprised of a numerous number of different types of cells that are well organized and have different roles in protecting the body against infections (Goldsby et al, 2003). The immune system can be classified in two main subsystems; they are termed as the innate/general resistance system and the adaptive system. An effective immune response continually involves both the innate system and the adaptive system.

The innate immune system or general resistance provides a first-line of defense against pathogenic agents. The response are however not only specific to particular pathogenic types. On the other hand, the innate immune cells are specific for all microorganisms. Thus it prevents the

innate immune system from involuntarily recognizing host cells and attacking them. Repeated exposure to the same pathogenic agent prevents their innate immune responses from improving. The innate immune system lacks memory to identify old pathogens.

An anatomic barrier such as intact skin and mucous membrane acts as the protective defense for the immune system which prevents the entrance of many microorganisms and toxic agents. The acidic environment of the skin prevents the growth of microorganisms. The microorganism that resides in the mucosa and the skin compete with each other for their nutrition and the attachment site. Apart from that, the mucus and the cilia on the mucous membranes facilitates in the trapping of microorganisms and eliminating them out of the body through the movement of cilliary staircase and the mucus (Goldsby et al, 2003).

The innate immune system comprises many physiologic barriers like fever, gastric acidity, lysozyme, the normal body temperature, collectins and interferon. Many microorganisms are inhibited in the normal body temperature and as the temperature of the body is increased as in fever many more microorganisms are inhibited. The acidic condition of the stomach is also responsible for killing many other microorganisms. Lysozyme, which is found in tears and mucous secretions, is a hydrolytic enzyme that can destroy the peptidoglycan layer of the bacterial cell wall thus killing those(Goldsby et al, 2003).

Interferons are produced by virally infected cells and contain a group of proteins that can easily bind with non-infected cells and produce a generalized antiviral state. Collectins are surfactant proteins that are present in lung secretions on mucosal surfaces and in the serum. By disrupting their lipid membrane, they can kill certain pathogenic microorganisms. They can also enhance the susceptibility indirectly to phagocytosis and this done by clumping of microorganisms (Hartshorn et al, 2002).

For defensive measures of the innate immune system the complement pathways are also involved. Complement system is a group of proteins synthesized by the liver as a part of immune response. These proteins are designated by the letter C. For example- C_3 which is usually the inactive form and is broken down to the active form C_{3a} and $C_{3b.}$ There are three different complement pathways. When IgM antibodies or certain IgG antibody subclasses bind to the surface markers/antigens on microorganisms the classical pathway is triggered. The deposition of complement protein, C3b, onto microbial surfaces triggers the alternative or properdin pathway and this does not require antibodies for activation. The attachment of plasma mannose-binding

lectin (MBL) to microbes triggers the third pathway or the lectin pathway and like the properdin pathway it does not require antibodies for activation. The three pathways combine together to form a common pathway leading to the formation of the membrane attack complex which can form pores in the membrane of targeted cells. The complement pathway is essential in the opsonization of particulate antigens to phagocytosis and is useful in triggering a localized inflammatory response (The Merck Manuals Online Medical Library, 2008).

The innate immune response can cause inflammatory response which is essential. The body's response to invasion by an infectious agent or an antigenic challenge, or any type of physical damage is characterized as inflammatory reaction. The inflammatory response is characterized by the cardinal signs of redness, heat, pain, swelling and loss of function and it allows the products of the immune system into the affected area or inflamed areas (Lycke, 2012).

The pattern recognition receptors or PRRs also contribute to the innate immune response and these receptors are not specific for any given pathogen or antigen, but they can give a rapid response to antigens. PRRs are associated with the cell membrane and that is why they are termed as membrane protein and also found in all the membranes of the cells in the innate immune system. Although there are hundreds of varieties of PRRs, all the genes of the PRRs are encoded in the germ line which makes sure limited variability in their molecular structures. Examples of PRRs include MBL, C-reactive protein, toll-like receptors (TLRs), NOD like receptors (NLRs), C-Type lectin, MX and pulmonary surfactant protein. The PRRs recognize PAMPs which are associated molecular patterns and can trigger cytokine release. Examples of PAMPs include LPS (endotoxin), lipoproteins (bacterial capsules), hypomethylated DNA (CpG found in bacteria and parasites), peptidoglycan (cell walls), flagellin (bacterial flagella) and double-stranded DNA (viruses). These antigens are not produced by human cells but by microbial cells. Complement activation, cytokine release, opsonization and phagocyte activation is a result of recognition of PAMPs by PRRs (Goldsby et al, 2003; Margolick, 2006;Janssens&Beyaert, 2003;Pashineet al, 2005).

Ultimately, the mononuclear phagocytes and granulocytic cells are also important to the innate response and form a link between the innate immune response to the adaptive immune response. Mononuclear phagocytes contain monocytes which circulate in the blood and macrophages that are present in the tissues. Antigen presentation, cytokine production, phagocytosis and antimicrobial and cytotoxic activities are dependent on both the monocyte and the macrophage

(The Merck Manuals Online Medical Library, 2008).For approximately 8 hours, the monocytes circulate in the blood and then migrate into the tissues and differentiate into specific tissue macrophages or into dendritic cells. There are several types of dendritic cells which are involved in different aspects of immune functions like the Helper T cells. The follicular cells that are found in the lymph follicle are involved in the formation of antibody-antigen complexes by binding in the lymph nodes (Goldsby et al, 2003; Pashine et al, 2005; The Merck Manuals Online Medical Library, 2008).

Neutrophils, eosinophil's, and basophils/mast cell are part of Granulocytic cells. Neutrophils are extremely active phagocytic cells and generally migrate to the inflammation site very fast. Eosinophil's are also phagocytic cells and are important for resistance against parasites. Basophil cells present in the blood and the mast cells in the tissues release histamine and other substances that are important in the development of allergies and take part in inflammatory response (Goldsby et al, 2003;The Merck Manuals Online Medical Library, 2008)

The innate system may solely be able to remove the pathogenic agent without any help of the adaptive system but the actions of adaptive immune system are specific to the particular pathogenic agent. This response will take longer to occur than the innate response. However, the adaptive immune system has memory which means that the adaptive immune system will respond to a particular pathogen more rapidly with each successive exposure which is not possible by innate immunity (Goldsby et al, 2003; Margolicket al, 2006)

B–cells/antibodies and T-cells are responsible for adaptive immune response. The T cells and the B cells are the two arms of the adaptive immune system. The T-cells compose cell-mediated immunity while the B–cells antibodies compose the humoral immunity or antibody-mediated immunity. Natural killer (NKs) cells are the ones that are only involved in innate immune responses (Goldsby et al, 2003;The Merck Manuals Online Medical Library, 2008).

The adaptive immune system has its first arm that is the humoral immunity which functions against extracellular pathogenic agents and toxins. B–cells are produced in the bone marrow and then migrate to the lymph nodes where they recognize the antigens, and this recognition is done without the need of antigen presenting cells or helper T cells. These antigens are called T-independent antigens as the T-cell activation is not necessary to activate the B–cells. Examples include dextran, lipopolysaccharide and bacterial polymeric flagellin. These antigens contain large polymeric molecules and can also induce numerous B–cells to activate the immune

response which is weaker and the initiation of memory is weaker than with T-helper cell activation (Margolick et al, 2006). In contrast, better immune response is found when the activation of B–cells with T-helper cell is done and with more effective memory, which is the ultimate goal for immune response. With the binding of the antigen to the Fab region on the B–cell receptor and secondary signaling from cytokines released by T-helper cells, the B–cells begins somatic hypermutation at the Fab region which further increases the corresponding fit between the Fab region and the antigen. The process then leads to the maturation of the B cells into plasma which is then able to produce effective antibody that best corresponds to particular antigens (Goldsby et al, 2003).

The stimulated B-cells, or the clones of B-cells new antigens will be formed that will be more specific in nature. These cells may develop into plasma cells producing antibodies or memory cells which will remain in the lymph nodes leading to the initiation of new immune response to that particular antigen. It occurs during the primary immune response when the immune system is first exposed to a particular antigen (Margolick et al, 2006).

The process of clonal selection and expansion usually takes several days to occur and it involves the production of IgM. During the primary immune response IgM is the first antibody that is produced.If the immune response progresses, the activated plasma cells will produce IgG that is specific to a particular antigen. IgM is the first antibody produced and is a large antibody with better neutralizing property. Apart from that, IgG binds more effectively to the antigen and aids in opsonization (The Merck Manuals Online Medical Library, 2008).

On the other hand, plasma cells produce other antibodies which are IgD, IgA, and IgE. IgD. These are primarily found in the surfaces of mature B–cells. While, IgA is the antibody that is found in location such as mucous, saliva, tears and breast milk secretions. IgE is also another antibody that is involved in allergic reactions and parasitic infections. However, IgG is the most important antibody for vaccines (Goldsby et al, 2003).

The primary immune response is responsible for producing the memory cells. Any succeeding exposures to the antigen will result in a more rapid and effective secondary immune response. The secondary immune response will be composed of IgG and the reaction would be more rapid and larger (Pashine et al, 2005).

Intracellular pathogens are the main target of adaptive immunity, cell-mediated immunity. T-cells mature in the thymus and are then released into the bloodstream. Generally there are two

main types of T-cells namely the CD4 cells and CD8 cells (The Merck Manuals Online Medical Library, 2008).

CD4 cells or T-helper cells only recognize the major histocompatibility complex (MHC) II protein and they have CD4 co receptor. The MHC II protein acts as a marker of immune cells and is found in all immune cells. CD4 cells are essential for antibody-mediated immunity and they help in the B–cells controlling of extracellular pathogens. There are two subsets of CD4 cells, Th1 and Th2. Th1 cells is responsible for promoting cell-mediated immunity while the Th2 cells help promote antibody-mediated immunity (Goldsby et al, 2003; Pashine et al, 2005).

CD8 cells or T-cytotoxic cells have the same function as CD4 and have CD8 co-receptor and only recognize the major histocompatibility complex (MHC) I protein. The MHC I protein acts as a marker of body cells and is found on all nucleated body cells except for mature erythrocytes. CD8 cells are essential for cell-mediated immunity and in helping control of intracellular pathogens (Goldsby et al, 2003).

The T-cells both can only recognize antigen that has been processed and presented by antigen-presenting cells which is not possible by the B cells. There are two types of antigen processing (Goldsby et al, 2003). The first type of antigen processing occurs with viral antigens and tumor cells. It involves the attachment intracellular antigens along with MHC I proteins on to the surface of antigen-processing cells. The other type of antigen processing occurs with bacterial and parasitic antigens and it involves attachment of the extracellular antigens along with MHC II proteins to the surface of antigen-presenting cells (Goldsby et al, 2003).

1.2. The Mucosa

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. The mucosal membrane comprise of very specialized mechanical and chemical barriers that prevents the entry of the foreign materials such as pathogens that is responsible for causing disease or facilitates their degradation. An important function of the mucosa is to prevent the colonization and the entry of the foreign particles and also to prevent any aggravated immune response that would be harmful to the organism. 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, 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 sub parts namely the bronchus associated lymphatic tissue (BALT), the gut-associated lymphatic tissue (GALT), and the nasal associated lymphatic tissue (NALT) (Dwivedy & Aich, 2011). The initiation of the antigen-immune response occurs in MALT (Mucosa associated lymphoid tissues). The MALT has a characteristic feature of being covered by numerous lymphoid follicles which covers various regions such as the intestinal tract and respiratory tract (this is also referred to as the bronchus-associated lymphoid tissue or BALT). There is absence of MALT in the genitourinary tract and it is due to the mucosa –draining lymph nodes. They are essential as they play an important role in adaptive immune response (Iwasaki, 2007; 2010). The organization and arrangement of the mucosal immune cells and tissues is different from that of the body's natural systemic immune system and this difference is evident in mucosal tissues.

1.2.1. Types of Mucosal Tissue

The mucosal tissue can be categorized into two types, namely- Type I or Type II mucosae. Type I mucosae are found on the surfaces of the lung and gut, whereas Type II mucosae are present on the surfaces of the mouth, cornea and throat. The difference in both Type I and Type II mucosae are recognized by the kind of epithelium, the transport mechanism of immunoglobulin A (IgA), the presence of organized lymphoid tissue (MALT) and the composition of the local immune cells. Type I mucosal tissue, can be further differentiated by diffuse lymphoid tissues which are mainly connected with effector responses (cell and immune response) from the classified lymphoid tissues where most mucosal immune responses are originated. Diffuse lymphoid tissue constitutes primarily out of lymphocyte that is present as intraepithelial lymphocytes in the mucosal epithelium. Moreover, various lymphocytes are available in the lamina propria, which is the connective tissue present beneath the mucosal epithelium. These include principally CD4+ T lymphocytes. Additionally, a lot of plasma cells are produced. These are actually B lymphocyte that produces antibodies in an abundant manner. Transudate IgG or locally produced IgG is the essential defensive immunoglobulin of Type II mucosal surfaces (Dwivedy & Aich, 2011). On

the contrary, IgA is the fundamental immunoglobulin in Type I mucosal tissues (Iwasaki A. 2010) and is secreted as a dimer across the mucosal epithelium using an active transport mechanism with the help of the Polymeric Ig Receptor (pIgR) (Kaetzel, 2005).

Secretory IgA (sIgA) is primary in mucosal effector function. It is to a great extent protease resistant and can in this way bind and kill pathogens or toxins in the gut even in the presence of active digestive enzymes. The pathogens that have been neutralized are eliminated from the body alongside other digestive wastes. A large amount of IgA is lost as a result of secretion and elimination of the digestive tract. IgA is one of the most abundantly produced antibodies in the body and hence it helps to maintain levels of adequate mucosal protection. In both the respiratory tract and the intestine, sIgA provides a barrier to all the pathogens; hence it generates a powerful IgA response which is an important goal for mucosal immunization (Iwasaki, 2007; Craig& Cebra, 1971).

The vaccines should be directed towards the main site of mucosal immune activation so that an effective immune response is induced. The various inductive site of the mucosal immune system includes the organized lymphoid tissue such as the tonsils in the upper respiratory tract, the appendix in the intestine and the Peyer's patches. Beneath the mucosal epithelium is the presence of these organized tissues. Smaller versions of these nodules are found as the bronchus associated lymphoid tissue in the larger airways and in the intestine it is scattered as isolated lymphoid follicles. The organized lymphoid tissues are similar to the peripheral lymph nodes but differ in a sense that they contain more B cells in comparison to T cells (Iwasaki, 2007; Craig& Cebra, 1971).

Peripheral lymph nodes depend on the activated dendritic cells (DCs) that move from peripheral sites to present antigens and prime lymph node resident lymphocytes (Niess et al, 2005). On the Contrary, antigen presentation and lymphocyte priming in Type I mucosal tissue can happen in the nearby MALT through the functioning of mucosal DCs (Rescigno et al, 2001) or epithelial microfold cells (M cells). For transport of antigen in the mucosal lumen M cells are used. The M cells are essential for the capture and transport of microparticles by transcytosis to underlying lymphoid tissues. The M cells differ from the typical mucosal epithelial cells phenotypically. M cells differs in the sense that they do not have an organized brush borders or cilia (Neutra, 1996; Rajapaksha & LoDD, 2010) and but do have a basolateral pocket that encourages direct contact with B cells and CD4+ T cells. Owing to the absence of brush border, M cells are efficient in

their surveillance of the mucosal lumen, from where they catch and transcytose virus and microorganisms and transport them to the underlying immune cells. The transcytosed antigens are given off either to follicular B cells to give their responses or to mucosal DCs that can present the antigens to T cells in the MALT or move to mesenteric lymph nodes. To summarize the M cells are important due to their key part in antigen transport over the epithelial boundary and hence is important for bringing about an effective immune response and which is why it is an ideal target for mucosal vaccines delivery (Rajapaksha & LoDD, 2010).

1.3. Mechanisms of Mucosal Protection

The mucosal immune system has been broadly classified into two type namely the innate immune system and the adaptive immune system. The innate immune system consists of different recognition molecule and the natural killer cell (NKs), while the adaptive immune system comprises of different antigen representing cells, T cells and B lymphocytes (Neutra, 2006). The mechanism of mucosal protection is summarized in Figure 1and is discussed in the following sections.

1.3.1. Mucosal Innate Immunity

Delicate epithelial barriers separate the mucosal membrane of the gastrointestinal, respiratory and urogenital tract from the outside environment. In the gastrointestinal tract, for instance, a solitary layer of epithelial cells joined by tight intersections confronts a complex luminal environment that is rich in microorganisms. Epithelia and their related organs, (for example, the salivary organs) produce nonspecific resistances including mucins and antimicrobial proteins (Neutra, 2006). In any case, foreign antigens and microorganisms often rupture the epithelial boundary and mucosal tissues are sites of exceptional immunological activity. In the intestinal mucosa, scattered lymphoid and antigen-exhibiting cells are especially abundant; it has been assessed that there are more antibody producing cells in the intestinal mucosa than in the spleen and lymph nodes consolidated (Brandtzaeg, 1999; Egmondet al, 2001). In the digestive tract, where microorganisms are numerous, epithelial cells, together with intraepithelial lymphocytes and fundamental phagocytic cells can adjust and dampen these signs to anticipate undesirable

responses to nonthreatening supplements and the ordinary intestinal flora that could prompt mucosal irritation (MacDonald,et al, 2005; Rakoff-Nahoum, 2004; Backhed et al. 2005). Accordingly, mucosal tissues are in a consistent condition of caution, yet they are adjusted to the vicinity of outside microorganisms and their items. Thus, antibodies that would create vulnerable responses upon injection into a sterile area, for example, muscle may not cause serious implications when given mucosally due to the fact that mucosal tissues have greater exposure to microorganisms.

Utilizing various ways the recognition of foreign bodies takes place in the mucosal surface. The cell-intrinsic method of recognition involves the dendritic cell recognizing pathogen associated microbial patterns (PAMPs) such as bacterial cell wall components (e.g. peptidoglycan, lipoteichoic acid) and different forms of nucleic acid such as the double stranded RNA and the high –CpG-content DNA which are uncommon in nature. This is done by utilizing the different pattern recognition receptors which is followed by the presentation of the pathogens either by major histocompatibility complex (MHC) class I pathway (through the rough endoplasmic reticulum) or by MHC class II pathway (through autophagy). T cells are activated by the costimulatory molecules and cytokines which are induced by signaling of the pattern recognition receptors (Brandtzaeg, 2009). The MHC class I is dependent on the availability of the antigens while the MHC class II pathway is dependent on the autophagy process. The cell intrinsic recognition is a useful in recognizing either of the pathogenic or infected cells. The pattern recognition receptors induces the secretion of interferons (IFNs) and the dendritic cell activating cytokines when its encounters an infected cell. This is then followed by MHC class I or II antigen presentation (Vajdy, 2008). If the toll like recognition is involved then it induces the costimulatory molecules and cytokines which are responsible for activating the T cells. When Toll-like receptors (TLRs) encounter a pathogen, it moves along with the pathogen into the phagosome where by the MHC type II pathway and the antigen processing and presentation occurs (Brownlee, 2007).

The mucosal immune system is unique as it has the ability to differentiate between a pathogenic and a non-pathogenic cell which is done by the pattern recognition receptors (Shaikh, 2011). A class of this pattern recognition receptor is the toll like receptors which are generally more

specialized antigen and adjuvant sensing molecules that are found in almost all mucosal antibody presenting cells, macrophages or dendritic cells which stimulates natural, non-specific barriers and thus help in the building up of adaptive immune response. Apart from recognizing the toll like receptor are involved in maintaining a steady environment inside the gut. Just like the toll like receptors, the NOD-like receptors and the Type C lectins are able to take immediate action when they encounter pathogens or when there is tissue damage by involving the innate immune cells like the macrophages or the neutrophils thus they trigger the tissue repair process and helps in switching to the adaptive immune mechanisms (Iwasaki, 2007).

1.3.2. Mucosal Adaptive Immunity

Different strategies are utilized by mucosal pathogens to infect people. A few pathogens, for example, *V. cholera* and enteropathogenic *Escherichia coli* cause disease by colonizing on the epithelial surfaces. Pathogens, for example, Rotavirus and flu infection contaminate the epithelium, though others, for example, *Shigella Flexneri* and *S. typhimurium* create local contamination in the lamina propria. Different pathogens, including HIV and S. Typhi utilize the intestinal mucosa as an organizing range for systemic spread of contamination. Security against such various dangers includes numerous resistant effector methodologies that work on both sides of the epithelial barrier as shown in Figure 1 (Neutra, 2006).

A vital characteristic for the mucosal adaptive immune response is the local reaction and emission of dimeric or multimeric immunoglobulin A (IgA) antibodies that are dissimilar to other neutralizer isotypes and are not subjected to deterioration in the protease-rich outside environment of mucosal surfaces. In humans, more IgA is generated than the various immunoglobulin isotypes consolidated (Egmondet al, 2001). High concentrations of IgA antibodies (more than 1 mg every ml) are present in secretions that are connected with mucosal surfaces in ordinary people (Kozlowski et al, 1997; 2002). The protease resistance of secretory IgA (sIgA) is a consequence of its dimerization and high level of glycosylation amid its amalgamation in mucosal plasma cells (Mesteckyet al, 2005). Furthermore, its association with a glycosylated part (the secretory segment) got from the epithelial polymeric immunoglobulin receptor (pIgR) that intercedes transport of dimeric IgA crosswise over epithelial cells to the lumen (Kaetzel & Robinson, 1991). sIgA has different roles in mucosal protection. It facilitates

the inhibition of antigens or microorganisms in the mucus, obstructing the direct contact of pathogens with the mucosal surface by a system that is known as 'immune exclusion'(Lamm, 1997). Then again, sIgA of the suitable specificity may block or sterically ruin the microbial surface atoms that mediate epithelial attachment (Hutchings et al, 2004), on the other hand it may intercept incoming pathogens inside epithelial-cell vesicular compartments in spite of pIgR-mediated transport (Kaetzel & Robinson, 1991; Lamm, 1997).Interstitial fluids of mucosal tissues that underlie the epithelial barrier contain dimeric IgA that is generated by nearby IgA-secreting plasma cells. This may avoid mucosal-cell contamination by mediating the transport of pathogens that have ruptured the epithelial barrier into the lumen through pIgR (Robinsonet al, 2001).Alternately by mediating antibody dependent cell mediated cytotoxicity (ADCC) that prompts the destruction of local contaminated cells (Egmond et al, 2001; Black et al, 1996)

Local IgG synthesis likewise can happen in the mucosal tissues after the administration of antigen or antibody to mucosal surface (Kozlowski et al, 2002;Mesteckyet al, 2005). This IgG, and additionally sIgA, could play a critical part in blocking disease by sexually transmitted pathogens at this site and has been demonstrated for contamination with herpes simplex virus type 2 in mice (Parr & Parr, 1997). In the human intestine, 5–15% of mucosal plasma cells generate IgG, however IgG is defenseless to corruption by luminal intestinal and bacterial proteases. In large intestinal secretions, for instance, IgG focuses are by and large 30 to 100-fold lower than those of sIgA (Kozlowskiet al, 2000). The IgG whether produced from the serum or not, possess the ability to kill pathogens that enter the mucosa and anticipate systemic spread.

It is often accepted that the mucosal or serum IgG diffuses crosswise over epithelial barriers and into secretions by Para cell spillage. Nonetheless, receptor-intervened IgG transport may likewise happen. Late studies have indicated that an IgG-particular Fc receptor (neonatal Fc receptor, FcRn) is communicated by epithelial cells in the intestine and respiratory tract and can stimulate IgG transport in both bearings crosswise over epithelial hindrances. In this manner, this framework may export IgG, and may likewise stimulate the uptake of antigens into the mucosa (Yoshidaet al, 2004). Moreover, another IgA specific receptor has been distinguished on apical surfaces of microfold cell (M cells) that can mediate uptake of luminal IgA into Peyer’s patches (Mantiset al, 2002). The immunological importance of these uptake systems has yet to be resolved, yet there is some proof that they may encourage the examining of luminal immune complexes by the mucosal immune framework (Yoshidaet al, 2004; Corthesyet al, 1996).

Cytotoxic T lymphocytes (CTLs) in mucosal tissues cannot counteract pathogen passage; however they may have an essential part in clearance or control of mucosal viral diseases. Immunologically dynamic mucosal tissues, for example, the intestinal tract, contain a lot of CD4+ T cells that are focuses for HIV. Thus, the intestinal mucosa turns into a repository of HIV contamination paying little respect to the site of starting viral passage (Veazey&Lackner; Brenchley, 2004). Both CTLs and antibodies inside mucosal tissues may add to preventing the establishment of such mucosal reservoirs (Belyakovet al, 1998).

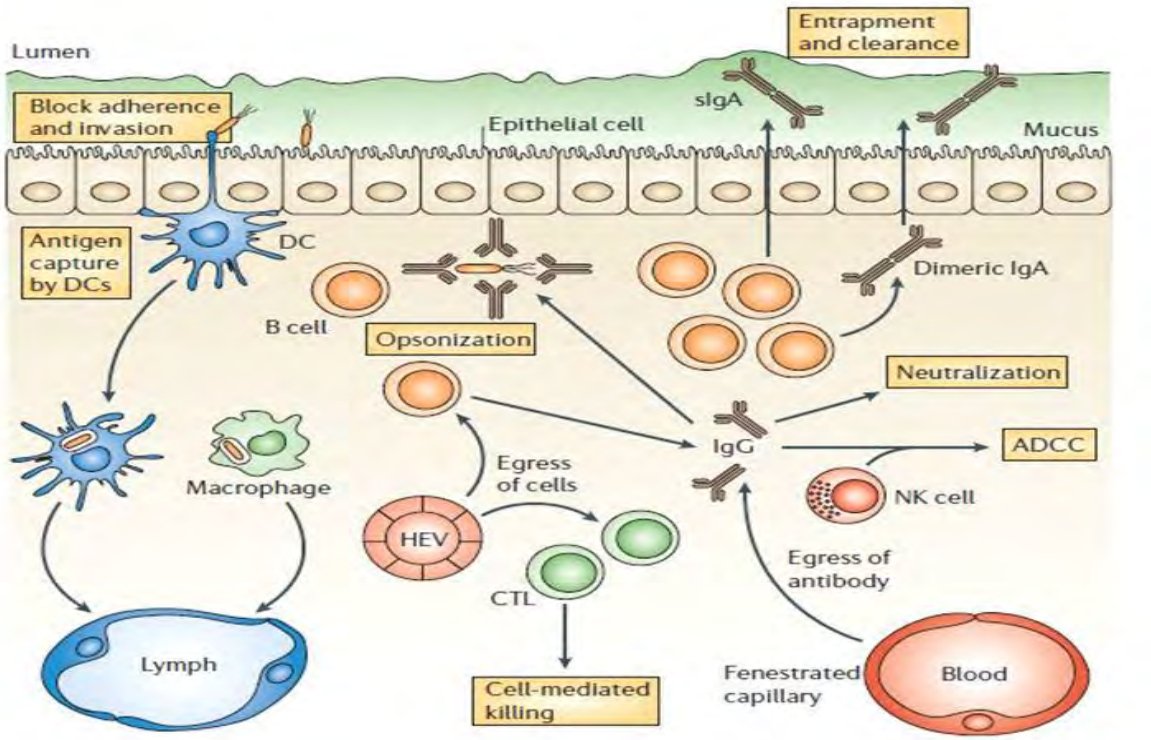

Figure 1: Mechanisms of immune protection at mucosal surfaces (adapted from Neutra and Kozlowski, 2006)
Various invulnerable effector instruments add to protection at mucosal surfaces. Antigen-specific effector B and T cells in the circulatory system perceive mucosal high endothelial venules (HEVs) and enter the mucosa. Mucosal B cells terminally separate to end up as mucosal plasma cells, the majority of which deliver dimeric IgA that is traded into discharges as secretory IgA (sIgA) to block antigens and pathogens, and to avoid mucosal attack. Killing IgG is additionally introduced inside mucosal tissues; mucosal IgG may be gotten from nearby plasma cells or from blood, by dissemination from neighborhood fenestrated capillaries. Contaminated cells may be executed by particular cytotoxic T lymphocytes (CTLs) or by antibody-dependent cell-mediated cytotoxicity (ADCC), cooperation between natural killer (NK) cells and antibodies. Pathogens can likewise be caught by dendritic cells (DCs) and macrophages, and conveyed to depleting lymph nodes.

1.4. Vaccines

Vaccines are biological preparations that enhance immunity against disease and either prevent (prophylactic vaccines) or treat disease (therapeutic vaccines) (Delany et al, 2014). The word 'vaccine' originates from the Latin word *Variolae Vaccinae* (cowpox), which Edward Jenner demonstrated in 1798 could prevent smallpox in humans (Delany et al, 2014).

1.4.1. Types of Vaccines

There are different varieties of vaccines available that are either being utilized or is being created for the purpose of prevention or treatment of infectious diseases. Under standard conditions, vaccines should be able to trigger the innate immune system and both arms of the adaptable immune system (NIH News, 2006). Regardless, different vaccines have their own advantage and disadvantages which only affects their ability to prompt immune response but also limits the usefulness of the vaccine types (National Institute of Allergy and Infectious Diseases, 2009).

Initially, it was shown that for diseases like measles, mumps, and chickenpox live or attenuated vaccines were used which were contained in the research center where the pathogens of the original disease that had been weakened in the laboratory. Hence, these vaccines were able to bring about cellular and antibody response and also produce long term immunity with just one or two dose of the vaccine. Typically, it is less hard to create live, attenuated vaccine with virus as opposed to other microorganisms such as bacteria due to the fact that virus has fewer genes hence it is simpler to control the viral attributes. Since, this type of vaccines contains live microorganisms care must be taken in their refrigeration process so that the potency of the organism is maintained and there are chances that the pathogen can revert back to its original form. Furthermore, we cannot use live vaccines for patients with weak immune system as it cause the actual disease. Examples of this type vaccine includes vaccines for Tuberculosis, Polio vaccine, Measles, Rotavirus and Yellow Fever (National Institute of Allergy and Infectious Diseases, 2009).

Inactivated vaccines such as the influenza vaccine are created by killing the pathogenic substance by means of chemicals, heat, or radiation. The vaccines are made more stable by the inactivation of the microorganism. These vaccines don't require any refrigeration and can be

freeze-dried for transport. Since, these vaccines produce weaker immune response; as a result there is a further need for booster shots for maintaining the immunity (National Institute of Allergy and Infectious Diseases, 2009).

While conducting experiments with mice *Raz et al*. showed that a vaccine produced from irradiated *Listeria monocytogenes* bacteria instead of heat killed bacteria gave protection in spite of challenges from the live bacteria. There was response shown in the T cells which was stimulated by the irradiated vaccine, earlier this stimulation was only shown when vaccines from live, weaken *Listeria* bacteria was used. Examples of this types of vaccine includes Polio vaccine (Salk vaccine), influenza vaccine, typhoid vaccine, cholera vaccine, plague vaccine and pertussis vaccine (NIH News, 2006).

Subunit antibodies are the ones that contain just epitopes (particular parts of antigens to which antibodies or white blood cells recognize and bind) that stimulates the immune system. Since these vaccines just utilize only a handful of antigens it decreases the probability of any adverse responses. However, this creates a problem in determining which antigens should be used for the vaccine. Examples of this types vaccine includes vaccines for tetanus, hepatitis B and diphtheria Toxoid vaccines are created by inactivating bacterial poisons with formalin. Formalin inactivates the toxin by interacting with them. The toxoid produces an immune response that act against the toxins. Examples of this types vaccine includes vaccines for include botulin and vaccines for tetanus and diphtheria (NIH News, 2006).

A conjugate vaccine is a different kind of subunit vaccines. In a conjugate antibody, antigens or toxoids from an organism are connected to polysaccharides from the outer covering of that microorganism to stimulate immunity (especially in case of children). Examples of this type of vaccine include vaccines for diphtheria and tetanus (NIH News, 2006).

Stripped DNA vaccines are still in the trial phases of improvement. These vaccines could utilize DNA for specific antigen to initiate an immune response. This DNA would be inserted into the body by injection from where the body cells would take up the DNA (NIH News, 2006).

Once the body cells have taken up the DNA then they could produce the antigen and could display them on to their surfaces which would stimulate immune response. DNA vaccines can have an advantage over other vaccines forms by creating an antibody response to both free antigens and the microbial antigens that are displayed in the cell surfaces. These vaccines would be easier and would also be less expensive to produce. Vaccines for influenza and herpes are still in the development stages (National Institute of Allergy and Infectious Diseases, 2009).

Recombinant vector vaccines are the vaccines that are still experimented. It involves utilization of an attenuated virus or microbes that would introduce the microbial DNA into the body cells. These viral antibodies would mimic a natural infection and thus cause stimulation of the immune system (National Institute of Allergy and Infectious Diseases, 2009).

There is a chance that the attenuated bacterium contains a genetic material for the antigens from a pathogenic microbe that has been inserted. These antigens from the pathogenic microorganism would then be shown on the harmless microbes thus mimicking the pathogen and causing the stimulation of immune response. The recombinant vectors vaccines that are made from both bacteria and virus for treatment of diseases such as HIV, rabies, and measles are in the test stages (National Institute of Allergy and Infectious Diseases, 2009)..

Apart from these vaccines, initiatives are taken to improve the vaccine adjuvants so they would target the innate immune system. These adjuvants could be categorized into two classes, either delivery system (for example, cationic micro particles) or immune potentiators (for example, cytokines or PRRs). In order to concentrate and display the antigens in a repetitious pattern and to assist the localizing antigen and immune potentiators and to target the antigens in the vaccine to the antigen presenting cells, the delivery system can be used. Likewise, to activate the innate immune system directly the immune potentiators can be used (Pashineet al, 2005)

1.5. Sampling of Antigens at Mucosal Surface

The presence of organized lymphoid tissue either within the mucosa or the draining lymph nodes is essential for the induction of the mucosal immune response against the pathogen or foreign materials, microorganisms and vaccines (Figure 2) (Egmondet al, 2001).The concentration of the microorganism is high in organized mucosal inductive sites (such as the lower intestinal tract) and they are the sites (such as the palatine or the lingual tonsils and adenoids in the oral and the

pharynx) through which the pathogens are likely to enter the body. Agglomeration of the organized mucosal lymphoid follicles in humans causes the Peyer's patches in the distal ileum and presences of numerous isolated follicles are found in the appendix, colon and the rectum (O'Leary et al, 1986). The overlying of epithelium is induced by the differentiation of a specialized follicle associated epithelium (FAE) containing M cells and is influenced by the presence of mucosal lymphoid follicles (Neutraet al, 2001). Intraepithelial pockets are formed by the M cells where the lymphocytes migrate and deliver samples the pathogens from the lumen and to the underlying DCs via the vesicular transport mechanism.

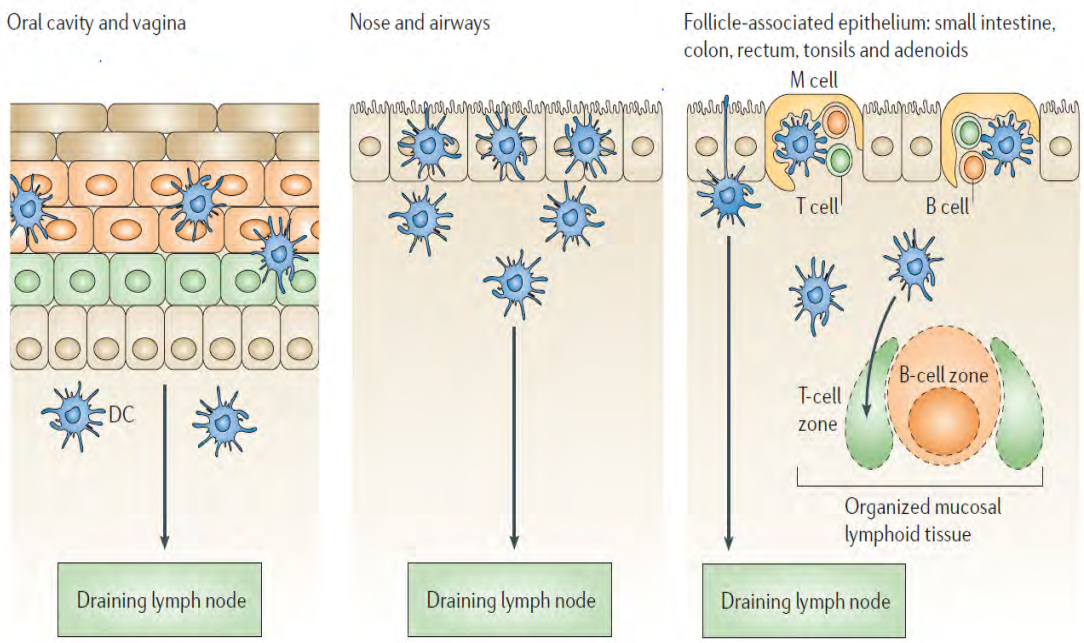

Figure 2: Antigen sampling at mucosal surfaces: collaboration of epithelial cells and dendritic cells (adapted from Neutra and Kozlowski, 2006)

Antigen sampling strategies are adapted to the various epithelial obstacles that cover the mucosal surfaces that are present throughout the body, but all involve collaboration with dendritic cells (DCs). DCs might congregate spontaneously beneath epithelia, migrate into the epithelial layer, and even cause the extension of the dendrites into the lumen in order to capture antigens. DCs from any mucosal surface could travel to the nearest draining lymph node to present antigen to T cells. At the sites of organized mucosal lymphoid tissues, specialized microfold (M) cells in the lymphoid follicle-associated epithelium supply antigens across the epithelial barrier straight to sub epithelial DCs that then present the antigen locally in adjoining mucosal T-cell areas.

The FAE is different from the absorptive epithelium in the intestine.Chemokines such as CC-Chemokine ligand 20 (also called MIP3α) and CCL9 (also called MIPIγ) are produced by the FAE of mouse small intestine Peyer's Patch. These cytokines can attract the lymphocytes and DCs that is responsible for expressing the CC-chemokine receptor 6 (CCR6) or CCR1, respectively (Iwasaki & Kelsall, 2000; Zhao et al, 2003; Hopkins et al, 2000) (Figure 2). High density of phagocytic cells at the site of entry of pathogen is due to result of the attraction of DCs to the FAE. This probably promotes the local antigen sampling and hence reduces the chances of local infection. The DCs in the subepithelial dome regions of Peyer's patch in mice capture the particles and the live bacteria that are transported across the FAE by the M cells. In the SED region (Hopkins et al, 2000) the DCs are phenotypically immature but once they capture the antigen they move to the T cells zones where they upgrade themselves into mature markers and MHC molecules (Shreedhar et al, 2003; Iwasaki, 2001). Sometimes the Peyer's patch DCs is able to carry the antigen to the draining lymph nodes where they form borders with the systemic immune system (MacPherson et al,1999).

Antigens and pathogens can be sampled on the mucosal surface through another type of epithelial DC collaboration in the absence of organized lymphoid tissues. Throughout the pseudostratified, stratified and simple epithelia the motile DCs are able to migrate into the narrow spaces between the epithelial cells and the outer sites of the epithelium from where they are able to obtain samples of the pathogens directly from the luminal compartment (Holt et al, 1990; Miller et al, 1992; Niess et al, 2005).Interaction between the local lymphocytes and the interepithelial or subepithelial DCs that have captured pathogens can potentially give rise to the memory response (Fagarasan & Honjo, 2003) or immune tolerance (Mayer& Shao, 2004). This means they could let them escape the mucosa through the lymphatics to present the antigens to native T cells of the draining lymph nodes. It has been found that different DCs have different roles in determining the type of immune response *in vivo* including those in the Peyer's Patch. But there is a need for more research about the migration pattern of the DCs; like the transcutaneous immunization results in mucosal immune response and it has been found that the DCs migrate to the Peyer's patch (Belyakov,2004).

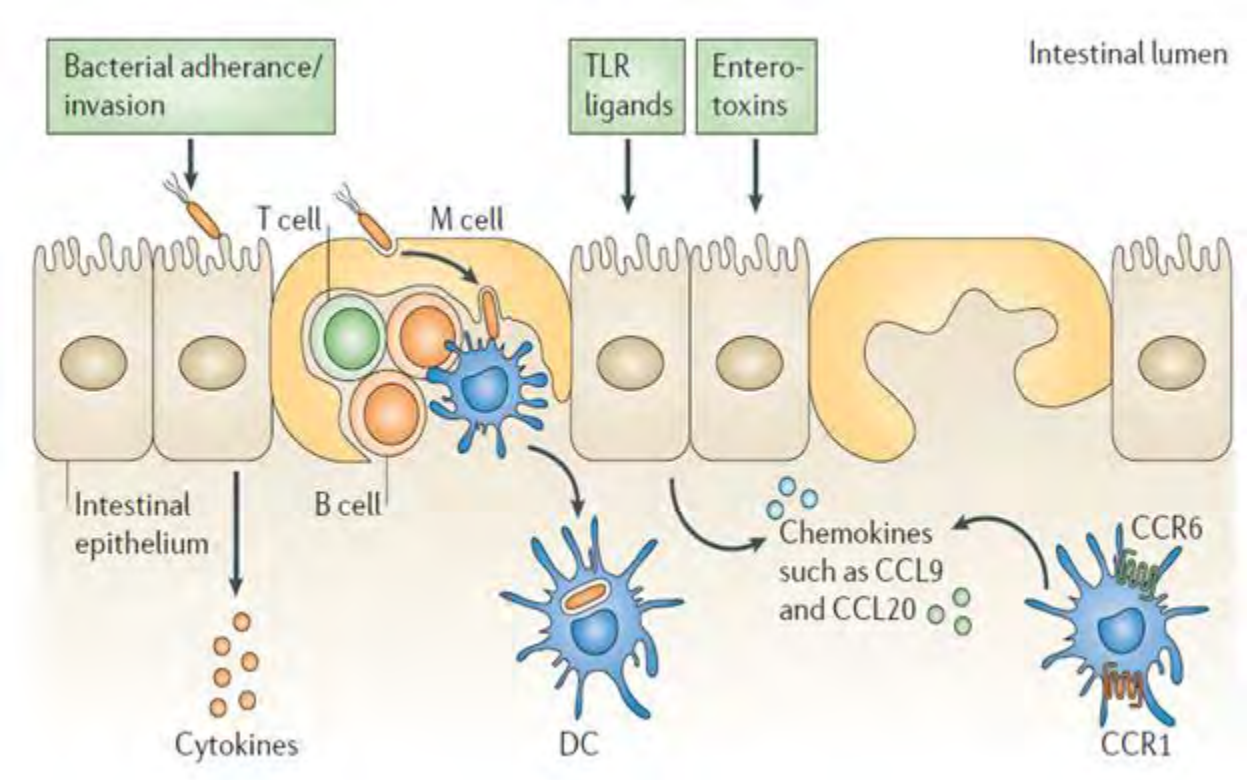

FIGURE 3: Functions of the Follicle-Associated Epithelium (adapted from Neutra and Kazlowski, 2006).

The follicle-related epithelium (FAE) involves microfold (M) cells which are specialised for endocytosis and rapid transepithelial transport of intact antigens and microorganisms into intraepithelial pockets that include B and T cells and coffee dendritic cells (DCs). Nearly all of FAE cells are enterocytes with apical microvilli lined with the aid of a thick brush border glycocalyx. FAE enterocytes don't transport antigens, however they could make contributions to antigen sampling by sensing luminal pathogens and their merchandise and releasing cytokine and chemokine signals that appeal to and spark off DCs. CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; TLR-Toll-like receptor.

1.6. Measuring Mucosal Immune Response

The design of effective mucosal vaccines and adjuvants will be easier if the immune responses that cause the mucosal protection can be identified. Lack of reliable and sensitive techniques have limited our identification process, hence making it difficult to develop new mucosal vaccine. The measure of the protective ability of most of the existing vaccines that are licensed is the induction of the antibody that functions in the opsonophagocytosis or the neutralization [65,

66]. The main goal of using vaccines in humans is the prevention of the mucosal replication of virus (such as influenza, polio and rotavirus) and bacterial pathogens (such as pertussis, typhoid) by means of locally synthesized IgA or transcytosed IgG which are present on the mucosal surface (Plotkin, 2001; 2010). The mucosal microorganism that are present at the time of interaction with the pathogen correlated are associated with the protection against the local colonization and is also involved with the systemic infection, as well as to provide protection against the disease that is caused by the invasion of the pathogens. However, it is difficult to measure the amount of antibodies that is associated with the protection by statistical method as large variations results from sample collection and dilution. As a result, there are studies which tests the protection offered by the known quantities of antibodies that is topically administered directly to the mucosa which includes measure of only available for the actual antibody concentration that is being induced by the vaccines. As a result serum antibody level is the best way to give a measure associated with the protection (Veazeyet al, 2003)

IgG antibodies plays vital functional roles which includes opsonophagocytosis, complement fixation, neutralization, inhibition of epithelial transcytosis or cell-cell transmission and also cell dependent cellular cytotoxicity which are also important in defining the humoral that is associated with the protection. It is often seen that the assays of antibody that are only based on antigen binding do not provide any information of the function and hence it necessarily does not does not correlate with the protection. For instance, when the children are injected with meningococcal unconjugated polysaccharide vaccines it gives a significant amount of ELISA detectable antibody response but provides very low protection (Peltolaet al, 1977). However, the level of bacterial antibody increases gradually with age in this case and the amount of serum (less than equal to 1/8) are considered safe for all serogroups (Maslankaet al, 1998; Hanekom, 2005). However, it is unlikely that there is relationship between the rapid and convenient measure of the level of antibody binding by ELISA and the levels of functional antibodies can be easily predicted by priori (Maslankaet al, 1998; Hanekom, 2005).

In spite of providing a reasonable surrogate marker of protective immunity, serum IgG cannot measure the role of IgA. In an experiment conducted on the IgA deficient mice, the results showed that the serum IgG could provide protection against the pathogens at the mucosal surfaces, but the effector properties of the sIgA could provide additional benefits of protection that was not tested for. Although IgA is protective at the mucosal surfaces, unlike IgG they do

not initiate any immune response. Serum level of IgA does not show any relation to the level to sIgA present in special mucosal tissue. Hence, even if humoral is associated with the mucosal protection which is benefitted from the sophisticated assessments of the functional assays in the context of the protection against the pathogens present at the mucosal surface. In addition, different assay techniques are required to focus on the specific local protective effects of IgA at the mucosal sites. Antibody response is the most direct and useful means of measuring the protective immunity whereas cellular immunity is responsible for both regulation of immunity as well as for providing direct effector response. Till date, the lack of information about the mucosal T cell quantity, quality and the duration in relation to the systemic T cell responses after the infection or the vaccination are creating problems that ultimately hinder the design of mucosal vaccines. A lot of examples can be seen, where T cells are being presented as an important part in the protection mechanism whose function is more than just helping the B cells to produce antibody. Taking example of the live attenuated strain of *Mycobacterium bovis* bacillus Calmette-Guerin, which is the only vaccine available for protection against the *M. Tuberculosis*, and in this the cellular immunity is considered to be the primary function for protection (Tanget al, 2008; Neutra & Kozlowski, 2006). Although antibodies play a vital role by enhancing the macrophage uptake by the *mycobacterium*, it argues that this effector function does not significantly contribute to the protection (Abebe& Bjune, 2009). The cytotoxic CD8+ T cells plays a vital role in contributing to significant cross reactive protection against the influenza variant strains when live attenuated influenza vaccine is used (for example- FluMist) (Sunet al, 2011). In considering another example, we see that the rapid Th17 response to the *Salmonella* infection is responsible either for the colonization of the intestine or for providing a barrier across the intestinal epithelium. Hence, modification of the T cell responses by the mucosal vaccination strategies can have an effect on the host defense (Liuet al, 2009; Godinezet al, 2008).

1.7. Mucosal Barriers to Vaccine Design

Present methods for vaccination focus on the systemic immune system and stimulate a weak mucosal immune response. Mucosal reactions are most effectively potentiated when the antibody is conveyed straightforwardly onto mucosal destinations. Then again, direct mucosal vaccination

has been considered troublesome. One of the challenges for the mucosal vaccination is that mucosal antibodies have a tendency to end up being weakened in mucosal liquids, and mass flow may constrain deposition onto the epithelium of the mucosal system (Godinez et al, 2008). Additionally, mucosal antibodies have the affinity to end up getting stuck inside the mucus gel and are hence degraded by the proteases. According to recent literature, it is mentioned that the mucosal vaccines may be more proficient if they were designed to mimic physicochemical properties of the pathogens, particularly in charge and size (Neutra & Kozlowski, 2006; Rajapaksaet al, 2010). Mixed bags of methodologies exist for the delivery and presentation of immune modulatory particles to the host immune system. Of these systems, those that will be powerful for mucosal vaccination will need to

- overcome the physiological barriers that are present at mucosal courses,
- focus on the mucosal APCs for preparing of antigens that would lead to particular T and B cell activation
- control the kinetics of antigen and adjuvant presentation so that a long lived adaptive immune response is promoted.

It would be quite a challenging task to develop mucosal vaccines that would increase the immunity without compromising with the safety. One of the methods to do so is the creation of antibodies that are based on the infectious virus or bacterial pathogens that have been rendered safe by attenuation or inactivation. Another method is to utilize subunit antibodies utilizing recombinant viral or bacterial proteins that can be rendered adequately immunogenic without stimulating any adverse reaction. A third and yet another promising method for mucosal antibody development are based on the particulate delivery systems that is intended to mimic the immunogenic properties of normal pathogens (Neutra & Kozlowski, 2006; Rajapaksaet al,2010).All these methods are discussed below with special consideration given to their application for the development of the mucosal vaccination.

1.7.1. Mucoadhesion and Mucus Penetration

The mucus is a highly viscous solution and is a heterogeneous micro-environment that provides a physical barrier to not only the pathogenic substances but also the mucosal vaccine delivery. For a mucosal vaccine to be more effective in nature the mucosal vaccine must be able to cross the

mucosal barriers as well as it should be able to prevent the inactivation of the antigens and the adjuvant in the harsh mucosal conditions and must be able to deliver the vaccines to the target cells. The viscosity and the pore size of the mucus can affect the can obstruct the diffusibility of different agents on the mucosal surface. Compare to the viscosity of water, the shear dependent bulk viscosity is much greater around 100-10,000 times more (Laiet al, 2007). The pore size of the mucus was found to be around 20nm to 200nm when the cervicovaginal mucus (CVM) was viewed under the electron microscope (Olmstedet al, 2007) However, recent measurement of CVM found the pore size can be as large as up to 50-1800 nm when view using the multiple particle tracking system. The pore size is comparatively large in case of humans but in spite of this fact the human immunodeficiency virus (HIV) and herpes simplex virus show reduced diffusibility in human CVM. Mixing of the virus and the mucin fibre as a result of hydrostatic and electrostatic forces is said to be the cause for the agglomerate formation of the mucus microstructure. This agglomeration hinders the diffusion by trapping the viruses. The mucosal transport of synthetic carriers with controlled size and the surface chemistry of the mucus provides and efficient insights for the development of the vaccines that are meant to be delivered by mucus. The mucus penetration is promoted by the hydrophilic nature of the vaccines and the net-neutral surface chemistry (Laiet al, 2007; 2009). On the contrary, the mucoadhesion is enhanced by interaction of the positive charged surface with the negatively charged mucus layer and thus prevent the diffusion. We can create a balance between the mucus penetration and the mucoadhesion which depends mainly on the thickness of the mucus layer and its residence time on the mucosal tissue. For instance, the thickness of the human CVM of about (~200 μm) and the clearance time of about 6-17 hours is favorable for the Nano carriers which have a wide range of diffusibility and adhesion property thus faster clearance time requires more mucus penetration. In order to increase the mucoadhesion and the mucus penetration we can involve the surface modification technique which has been reckoned to be beneficial. Owing to the presence numerous hydrogen bond forming groups several natural materials such as chitosan, alginate, and derivatives of cellulose shows strong mucoadhesive properties (Smart, 2005). Rajapaksa *et al*. mentioned that coating the surface of poly (lactic co glycolic) acid nanoparticles with poloxamer 188 (which is a surfactant) increase the diffusivity and uptake of the nanoparticles by the mucosal surfaces. Polyethylene glycol can be used to enhance the mucus penetration or the mucoadhesion on the basis of the molecular weight. Nanocarriers that are attached with PEG

chains (≥ 10 kDa) are generally mucoadhesive in nature whereas those attached with shorter PEG chains (≤ 2 kDa) have greater mucosal diffusivity(Lai et al, 2009). The mucus binds with the high molecular weight PEG causing interpenetration and secondary interaction which promote the adhesion. For effective transport of the vaccines that are delivered using the mucus the concept of mucoadhesion and the mucus penetration will play vital role (Lai et al, 2009).

1.7.2. Targeting Epithelium, Microfold Cells or Dendritic Cells

Encapsulation of the vaccine with polymer-based particles can improve the delivery of the vaccines to their target sites. If the vaccines are not delivered properly to their target sites then they would not be able to give any immunological functions for which they have been designed for. Since mucosal DCs are the encounters the pathogens at first instance at the site of the entry point, numerous ligands have been examined to target these specialized APCs. Focusing on APCs, particularly DCs, was initially pioneered by the experiments of Steinman and Nussenzweig (Steinman & Cohn 1972; 1973; Nussenzweiget al, 1980) who were the first ones to recognize the receptors expressed particularly by these cells. The C type lectins are a group of calcium dependent lectins which were expressed on the surface of the DCs. There are various ways to target these receptors using natural ligands for the receptor, which includes examples such as the sugar mannose and mannon or antibodies that are directed against the receptor. The examples of the methods of the DCs involve the well-characterized DC receptor CD205 (also known as DEC205), the mannose receptor or DC-specific intercellular adhesion molecule 3-grabbing non integrin (DC-SIGN). Langerin is a type of receptor that is found in highly special intraepithelial DCs called the Langerhans cells that are found in the type II mucosae. The expression of the receptor vary not only on being either immature or mature DCs but also among the mucosal DCs subtypes that are present in both type I and type II mucosae. The intracellular directing of the receptor and thus the antigen presentation pathways are also characteristics of the targeted receptor. For the successful design of DC-based mucosal vaccines all of these factors must be taken into account. However, targeting the APCs is not a new technique to the mucosal vaccination but it could help create a strong immune response to the antigens that are delivered through the mucous pathway. The mucosal epithelial provide another prospect for targeting mucosal vaccines. The neonatal Fc receptor, FcRn, is available on some type of mucosal

epithelia and is also included in the transepithelial transport of IgG (Yoshidaet al, 2004). Galactosyl ceramide is an advanced glycosphingolipid found in the apical films of mucosal epithelial cells of the endocervical, rectal, andgastrointestinal mucosae (Alfsen et al, 2002). Another technique that has been utilized to enhance the adhesion between mucosal epithelium and the vaccine delivery system is to utilize high-affinity targeting ligands against M cells. In any case, few M cell receptors can be focused for vaccine delivery. Without a doubt, some receptors have been solely recognized only for the mouse and not for human M cells, thus restricting their helpfulness in clinical settings. *Clostridium* perfringens enterotoxin (CPE) has been recognized as a high-affinity ligand for the M cells. In particular, CPE binds Claudin-4 communicated on the apical surface of mucosal M cells.

1.7.3. Kinetics of Infection

Timing the antigens and adjuvants presentation to match the kinetics of pathogen infection is also predominant to the design of protective mucosal vaccines. The key for induction and renovation of protective viral immunity is deemed to be associated with the kinetics of primary rapd viral replication adopted by the use of virus elimination to low phases (Zinkernagel, 2003; Masopust & Ahmed, 2004). Attempts to mimic the kinetics of infection have shown that the time of adjuvant and antigen give can have profound effects on the resulting T and B cell response (Bachmann&Jennings, 2010). For example, there have been more CD8 +T cell responses observed when antigens are co administered with adjuvants equivalent to α-galactosyl ceramide (Fujiiet al, 2003), TLR ligands (Van Duinet al, 2006), or CD40-detailed antibodies (Bonifazet al, 2004). On the contrary, the delivery of adjuvants too early or too late after the antigen supply can impair antigen cross penetration (Dattaet al, 2003;Wilsonet al, 2006). The choice to deliver the antigens separately or in combination, either mixed or physically conjugated with the adjuvant additionally affects their colocalization in the phagosomes and also effectivity for presentation by the utilization of the APCs. Blander and Medzhitov demonstrated that the DC maturation and major histocompatibility complex (MHC) class II presentation come up only when the TLR ligands and antigens co-localize within the same phagosome. According to a recent study conducted by Kasturi et al. they observed that enhanced antibody titers and germinal center formation had been brought on when the antigens and adjuvants were delivered separately in

biodegradable nanoparticles. A couple of reports demonstrated that the antigen exposure in mice for a longer time can effect in co-stimulation of independent T cell responses and long lived T cell memory. Cranage et al. had recently validated that repeated vaginal administration of HIV-1 gp140 without an adjuvant can no longer induce serum and mucosal antibody responses (Cranageet al, 2010). In spite of the fact that the vaccine schedule and the coordination of the vaccine and adjuvants is well known to us, we still have little knowledge to figure out about how these parameters will be translated into effective vaccines.

1.7.4. Relationship between Commensal Microbes and Mucosal Immunity

The main intention of the mucosal immunization is to provide a protective immune response against pathogenic microbes in the lumen; however mucosal tissues are also abundantly colonized via non-pathogenic microbes that are referred to as the commensal microbes. Even though they are often considered as symbiotic, the commensal microbes may also be responsible for causing diseases if they show up in the wrong part of the body or if they overgrow the mucosal tissue. As a result, delivering a clear definition of what constitutes the commensal microbes is complicated. Indeed, the mucosal immune system is most likely to generate an immune response that is equivalent to sIgA antibodies towards the numerous microorganisms that might be or shall else be considered commensal (Macphersonet al, 2000). Commensals can also have a big impact on mucosal immune regulation and is usually considered as a factor that can have effect in the mucosal vaccination. Even though the studies on the subject of the mucosal microbial communities continues to be in its early stages, just a few occasions of the effect of commensal microbe on mucosal immunity have been recognized. For example, the intestinal epithelium recognition of microbes by means of TLR2 signaling is noted to be important to epithelial integrity and homeostasis, on the grounds that it regulates tight junction development (Cario et al, 2004; Rakoff-Nahoum et al, 2004). Direct results on immune regulation have also been described. In one case, a polysaccharide that was produced by the commensal *Bacteroides fragilis* was immediate to regulated development of regulatory T cells (Round&Mazmanian, 2010; Mazmanian et al, 2005). Moreover almost always, the manipulation of intestinal microbes in a tremendously restrained flora altered the connection among lymphocyte populations within the intestine, with CD8+T cells–mediated reductions in

numerous regulatory cell types (Weiet al, 2008; Fujiwaraet al, 2008). For that reason, the colonization of mucosal sites by means of commensals and their balance different pathogenic microbes can play a vital role in defining the effectiveness of mucosal vaccines. This is almost an important aspect within the world, where excessive influences of intestinal pathogenic bacteria and parasites on T cell regulation can override the planned effects of the vaccines and adjuvants (Weiet al, 2008; Fujiwaraet al, 2008).

1.7.5. Immunity versus Tolerance

Mucosal vaccine formulations have the advantage of being needle-free administration, however other reasons add uncertainty to the development of dosing and formulation approaches. For instance, whereas many pharmacologic agents require strict manage overdose supply with precise targets for plasma stages, an “ideal” target-delivered dose of vaccine will not be easily recognized. For a strong immune response may additionally require one or more booster doses to increase the initial primed response and solidify memory immune responses. Accordingly, the dosing is very complicated as they have to set up the dosing and timing of booster shots to induce adequate protecting immunity within the greatest percentage of recipients. In the case of mucosal delivery, successful delivery of the vaccine to the target immune cells is complex by the variability in the uptake mucosal environment, by the ever changing requirements for booster doses and through the superimposed immunological effects of ongoing illness or infection.Although the intention of mucosal administration of vaccine is to induce protective immunity, a well-recognized phenomenon that is termed as the oral tolerance or mucosal tolerance is associated with mucosal administration of antigen (Weiner et al, 1994; Whitacre, 2000). Indeed, some target vaccination methods give immunological tolerance in place of the immunity; this happens mainly with protocols wherein an immunological adjuvant isn't provided. In both T and B cell responses this phenomenon applies, even though in some cases a T cell's response coexists with B cell tolerance due to a split tolerance. The immunological tolerance induction, mucosal or otherwise, will depend on the dose and timing of antigen delivery. Low-zone tolerance is caused with the help of low doses over a prolonged period of time, whereas the high-zone tolerance is brought about with the help of an excessive dose that overwhelms the immune method. The mechanisms invoked have variably involved the central or

peripheral deletion of reactive cells and activation of regulatory T cells, but in all instances they depend upon the active recognition of the antigen by means of the immune cells. Mucosal vaccine supply formulations need to take these prospects into account. Low-dose extended release delivery of the antigens may be useful for pharmacologic agents. However for vaccine antigens the slow release could lose the benefit of adjuvant activity and as a result can induce tolerance. On the contrary, fast delivery of a high antigen dose could have the possibility to induce a high-zone tolerance. For that reason, various controlled release designs which are described below are required to be considered, so as to design optimum antigen release kinetics (Weiner et al, 1994; Whitacre, 2000).

As in case of the autoimmune disorder or an allergen immunotherapy tolerance induction (also known as the immune deviation) would, in some instances, be an intentional goal. Sublingual administration of allergens is being explored, despite the fact that delivery of these formulations is easy (Radulovic et al, 2011). In clinical practice, one theoretical complication is concurrent contamination, and associated innate immune activation that might have the ability to provide adjuvant activity. Nonetheless, allergen immunotherapy is most commonly used within the setting of existing immunity (hypersensitive reaction), so abrogation of intended tolerance induction is less possible. It's not known if preventative tolerance induction is more likely to risk immune induction instead of tolerance if administered within the presence of mucosal infection (Radulovic et al, 2011).

Chapter-2

RESEARCH METHODOLOGY

2. Research Methodology

In this article, the current status of mucosal delivery of vaccines is summarized. This review is based on a literature search carried out using the "Web of Science" (ISI). The keywords "Mucosal vaccine" and "vaccine delivery" gave 1517 hits for the period from 1985 to 2015. The results were cross checked by searching Elsevier's "Science Direct", PubMed, SpringerLink, and Informa world. Other references and conference proceedings have also been included. At present there is a great interest in developing mucosal vaccines against a variety of microbial pathogens as most pathogens access the body through the mucosal membranes. Therefore, effective vaccines that protect at these mucosal sites are much required. However, the size of the mucosal tissue provides a formidable challenge to inducing protective immunity at the natural portals of entry of pathogens. Moreover, the development of mucosal vaccines, whether for prevention of infectious diseases or for oral-tolerance immunotherapy, requires efficient antigen delivery and adjuvant systems. Such systems must protect the vaccine from physical elimination and enzymatic degradation, target mucosal inductive sites including M cells and appropriately stimulate the innate immune system to generate effective adaptive immunity especially for vaccines against infections. Significant advances have recently been made in the development of improved mucosal vaccines delivery systems. It has been found that antigens delivered in particles (particulate drug delivery systems) are better recognized by the innate immune system and are stronger inducers of mucosal immune responses compared to soluble antigens (live-attenuated vaccines and subunit and conjugate vaccines). Novel mucosal adjuvants with prospects for human use have also been introduced. The purpose of this review is to discuss several different approaches to induce mucosal immunity to vaccines emphasizing on mucosal tissue targeting and new immunization routes with a particular focus on the delivery systems.

Chapter-3

RESULTS AND DISCUSSION

3. DESIGN STRATEGIES FOR MUCOSAL VACCINES.

The promise of mucosal vaccines is that they can generate local immune responses, resulting in the protection that might not be achieved following natural illness; this is done by going over the earliest cellular interactions with nearby APCs and mucosal follicle cells. Nevertheless, directing vaccines to mucosal tissue with administration through the correct mucosal routes is unlikely to be enough for eliciting protective mucosal immune responses. Ideally, mucosal vaccines target the inductive sites in mucosal tissue and thus, promote interaction with local cells of interest. The size, architecture, and floor chemistry of specific carrier methods can also be manipulated to maximize interactions with immune cell objectives (Table 2). The following discussion is focused on the design standards which can be valuable in developing novel mucosal vaccines. To be more precise a number of delivery techniques that can be utilized to target vaccines (both antigen and adjuvant) to correct cells of the mucosal immune systems and in addition that can be used to make proper and optimal immune stimulation.

Particulate carrier type	**Vaccine characteristics**	**Advantages**	**Disadvantages**
A. Emulsions			
Water in oil emulsions	Th1-stimulating antigens	Slow release of antigen	Reactogenicity
Oil in Water emulsions	Th2 stimulating antigens	Slow release of antigen	Reactogenicity
B. Liposomes	Water soluble drugs Water insoluble drugs DNA Proteins	Easy surface modification Synthesized from non-toxic material Dual function Wide range of antigen encapsulation	Low antigen loading Low stability
pH-sensitive	DNA cytotoxic	Efficient Endocyclic	Intramembrane repulsion

liposomes	agents Proteins	release	
Cationic liposomes	DNA siRNA	Controlled release of antigens	Non-specific interaction
C.Synthetic polymers			
PLGA	Plasmid DNA Protein Peptide Low molecular weight molecules	Controlled release Sensitive to environment Stable microenvironment Biocompatible	Low loading efficiency Degradation of antigens during encapsulation
PLA	Plasmid DNA Protein Peptide Lipophilic compound	Controlled release Surface easily modified	Low loading efficiency Degradation of antigens during encapsulation
PEI	Plasmid DNA	Efficiently transfected	Cytotoxicity
D.Virus like particles	Plasmid DNA Protein Peptides	Lacks viral genes Highly immunogenic High rate of uptake Undergoes self-assembly	Formulated by recombinant technology

TABLE 2: Particulate carriers commonly employed to deliver vaccine antigen to mucosal sites (adapted from Woodrow *et al*, 2012)

3.1. Live-Attenuated or Inactivated Vaccines

Live attenuated vaccines are composed of live bacteria or viruses which might be made so much less virulent than the pathogenic parental bacterial or viral traces. The important function of the live attenuated vaccines is that they are capable of providing high level of antigen exposure and to some extent also their adjuvanticity is inbuilt (Manicassamyet al, 2009). The live bacteria and viruses can be engineered to act as vaccines vectors and hence they can carry the antigens from other pathogens. However, vaccine vectors could also be much less effective than are living attenuated vaccines due to the presence of preexisting vector‐specific immunity that would minimize vaccine efficacy, as seen in case of adenovirus vectors (Tuckeret al, 2008).

Ideally, the live attenuated vaccines cause a slight infection at the site of administration. They are able to also be engineered to have a restrained capability for replication and to give a sufficiently high antigen load at the site for immunization to be effective, even as averting unwanted local inflammatory responses (Pasettiet al, 2011). These attributes are predominant on account that prolonged antigen exposure and a significant amount of antigen are commonly required for the induction of mucosal immune responses, mostly in case of oral vaccination (Lycke, 2012).

An important challenge for the development of the live attenuated vaccines is to attain the balance between sufficient attenuation and vaccine immunogenicity. For instance, the efficacy of the live attenuated vaccines that operate against rotavirus contamination were made less infectious by constantly passing the virus in the host cells cellphone cultures (Tucker et al, 2008; Greenberg et al, 2009).These vaccines result in most effective in intranasal and sublingual vaccines against Bordetella pertussis (the pathogen responsible for causing whooping cough, rotavirus, influenza virus, measles virus and norovirus (Pasetti et al, 2011; Li et al, 2011; Simon et al, 2011; Tribble et al, 2010)

3.2. Subunit and Conjugate Vaccines

They are the second largest category of licensed prophylactic vaccines and are based the pathogen-targeted proteins or based on polysaccharides conjugated to proteins or peptides. This class includes the toxoid vaccines which are most commonly isolated and inactivated bacterial toxins designed to elicit the immunity to the toxic compounds (as an alternative to the live

microbe) that is responsible for causing the disease. The diphtheria toxin vaccine and tetanus toxin vaccine which can be administered in blend during childhood are all examples of toxoid vaccines. Subunit and conjugate vaccines are also a category of toxoid vaccines that are administered principally by means of subcutaneous or intramuscular routes but are not administered through the mucosal route, and the serological correlates of antibody immunity have been established for these vaccines only(Woodrow and Kalia, 2012). One exception to this is the Dukoral (registered by Crucell, Netherlands) vaccine, which is used against cholera and is composed of the cholera toxin B subunit and the inactivated strain of *Vibrio cholera* O1. Oral, but not parenteral, immunization with inactivated whole-cell cholera bacteria alongside the cholera toxin B subunit protects towards cholera colonization and toxin binding, respectively. The orally administered vaccine induces protective mucosal IgA antibodies towards the bacterium and its toxin, and presents long-lasting intestinal immunological memory. There are not any other examples of efficaciously of the licensed subunit vaccine that are administered through the mucosal immunization and are able to provide protection (Woodrow and Kalia, 2012).

3.3. Particulate Delivery System

Despite the small number of licensed subunit and conjugate vaccines for the mucosal immunization, several promising studies have highlighted the potential and the value of this approach for mucosal vaccine development. Lo *et al*. have examined the intra-nasal administration of soluble influenza hemagglutinin protein, which is functionally linked to a focusing on peptide that's distinct for Claudin-4 on M cells. They have found that this soluble fusion protein was once capable of inducing both a specific serum IgG and a mucosal IgA response. In a latest study conducted by Ye, Zhu and coworkers(Yeet al, 2011), a subunit vaccine that consisted of the HSV-2 envelope glycoprotein and fused to the IgG Fc fragment was once delivered intra-nasally; it elicited systemic as well as mucosal B and T cell responses and thus provided protection from intravaginal challenge with HSV-2. In addition, repeated intravaginal immunization of recombinant HIV-1 without using a mucosal adjuvant induces systemic and mucosal neutralizing IgG antibodies (Wilsonet al, 2006). Yet another pleasing area in subunit vaccines has emerged recently which utilizes the proteins of outlined structures such

as the scaffolds for the presentation of immunogenic epitopes (Burton; Correia et al.; Ofeket al, 2010). Epitope scaffold vaccines have been rationally designed for HIV-1 and surrounds a protein gp41; epitopes known to elicit greatly neutralizing antibodies (2F5 and 4E10) (Correia et al.; Ofeket al, 2010). Some epitope scaffold designs had a thousand-fold-more binding affinity for their neutralizing antibody and firmly based that epitopes grafted onto the backbones of proteins can probably function as the immunogens for antibody generation (Correiaet al, 2010).
Mucosal immunization with subunit vaccines has detailed challenges associated with not simplest the immunogen but also the route of antigen supply. Mucus does do not cause any hindrance to the diffusion of soluble proteins, and transport of gigantic protein antigens must be viable (Saltzmanet al, 1994). Nevertheless, mucosal immunization with protein antigens is constrained in order to guard the protein antigens from degradation by means of the mucosal proteases or commensal micro flora. In addition, subunit vaccines are most of the time poorly immunogenic and require the usage of adjuvants to be powerful. Few adjuvants are accredited for human use, and reliable and powerful mucosal adjuvants are required to make the subunit vaccines more effective. Vehicles that are engineered with correct mucosal adjuvants to protect and deliver antigens. In the next sections, we will discuss about using particulate delivery methods for the development of mucosal vaccines (Woodrow & Kalia, 2012)

3.3.1. Virus-Like Particles and Virosomes

Virosomes and Virus-like particles (VLPs) represent a class of subunit vaccines where the immunogens are derived from viral add-ons that self-assemble into 3-d architectures that preserve the antigenic constitution of virus immunogens. VLPs are shaped as a result of self-assembling of a number of viral capsid or envelope proteins which might be expressed recombinantly in mammalian or insect cells. The hepatitis B vaccine was the first commercially conceivable VLP-centered vaccine (Loweet al, 1997). The Hepatitis B vaccine was made from the self-assembling of the hepatitis B floor antigen (HBsAg) that is expressed recombinantly in the yeast cells. Another VLP that has been licensed for human use is the human papillomavirus (HPV) vaccine. The quadrivalent HPV vaccine from the studies by Merck & Co. (registered by Gardasil) consists of the L1 capsid proteins of HPV-6, -11, -16, and -18 forms which might be self-assembled into VLPs (Wheeler et al, 2009) and expressed recombinantly in yeast. The

mechanism of protection by which the VLP vaccines for HPV just isn't fully understood, and no immune correlates have been associated directly to safety. The protection is posited to be as a result of serum-neutralizing IgG that transudes throughout the cervical epithelium in sufficient amount enough to bind HPV virions and restrict infection (Jansen& Shaw, 2004; Lowe et al, 1997; Stanleyet al, 2006; Fife et al, 2004). Licensed VLP vaccines like subunit vaccines presently require the co-administration with adjuvants so that it is more effective.

Virosomes can also be considered as a special class of liposome vaccine delivery techniques whereby viral membrane proteins are built-in into unilamellar vesicles composed of viral and other artificial or natural lipids (Felnerova et al, 2004).The most evolved virosomal systems are based on those lipids that are derived from viral, egg, or from the synthetic lipids and membrane proteins of influenza virus; combined they are referred to as the immunopotentiating reconstituted influenza virosomes (IRIVs). A unique feature of IRIVs is the presence of the influenza-derived proteins neuraminidase and hemagglutinin, which helps to distinguish these virosomes from all different lipid-based particulates similar to that as the liposomes. EpaxalR (manufactured by Crucell, Netherlands) is a virosome vaccine that is used for the treatment of hepatitis A and it is prepared by the adsorption of formalin-inactivated hepatitis A virus onto the IRIVs (Zubriggen et al, 2000). Inflexal is a trivalent influenza vaccine that is based on a blend of three monovalent virosomes that is reconstituted from fluctuatent influenza virus strains (Gluck et al, 1994; Mischler & Metcalfe, 2002). Preexisting immunity against influenza is a must needed feature that is needed for the adjuvant activity of IRIVs. In a contemporary novel, Bomsel et al. evaluated the protective efficacy of IRIVs grafted with HIV-1 gp41 subunit antigens. They demonstrated when their HIV-1 gp41 subunit IRIV is administered in two doses intramuscularly followed by two doses intranasally resulted in full protection against the vaginal simian-HIV challenge, whereas four intramuscular doses resulted in a half or 50% of the protection (Bomsel et al, 2001; McElrath, 2011). Antigen-specific IgA antibodies that are present in the protected animals prevented HIV-1 transcytosis and IgG antibodies while in the meantime giving a neutralizing or an antibody-dependent cellular cytotoxicity. Incredibly, these animals that lacked the serum-neutralizing antibodies suggest that the vaccine-induced mucosal antibodies may be principal cause for the immunity against HIV-1 (McElrath, 2011).

Despite of a limited quantity of VLP and virosome vaccines for human use, they provide us an exciting platform for the development of novel mucosal vaccine technologies. Both virosomes

and VLPs are small in size and the composition of their surface chemistry will also be designed to minimize hydrophobic and electrostatic adhesive interactions with mucus. VLPs can be produced from a numerous exceptional expression systems. They can also be engineered for recombinant expression of multiple antigenic epitopes and for the incorporation of co-stimulatory and immune-regulatory proteins. Nevertheless, VLP technology can also be limited by means of difficulties of scale-up, the requirement for adjuvants and the need for purification from the expression systems. Most effective virosomes have been used easily without addition of further adjuvants (Moser et al, 2007), hence they could also be appealing as mucosal vaccines. Despite the fact that both VLPs and virosomes have the potential to be used as vaccine carriers, they are often more difficult to formulate and are less reproducible when compared with the artificial polymer nanoparticles (Woodrow& Kalia, 2012).

3.3.2 Non-viral, Polymer-Based Carrier Systems

Polymer-based micro- and nanocarrier methods are a developing technological platform for the design of novel mucosal vaccines. In latest years, we've observed revolutionary techniques that have been to engineer these carriers to overcome mucosal limitations so that vaccines and adjuvants are delivered to the oral, nasal, and anogenital mucosae. The carriers may also be made of a kind of substances—equivalent to natural or artificial polymers, proteins, lipids or inorganic substances—to prepare particles and drugs of a controlled size and structure. Nanocapsules represent a reservoir delivery system from where the vaccines may be enclosed inside an aqueous or oil-based core and also surrounded by an outstanding or semi-solid material shell (Woodrow & Kalia, 2012).. On the contrary, nanoparticles are solid particles from where the immunogens are dispersed inside the polymer matrix or is adsorbed to the particle surface. These carrier technologies can also be designed to supply low-molecular-weight compounds as well as biologics (such as peptides, proteins, nucleic acids) that upon particle degradation, erosion, swelling, or diffusion from the polymer matrix becomes bioavailable .Another reason for selecting biomaterials is their biocompatibility and their capacity to be engineered for tissue and cell targeting (Woodrow & Kalia, 2012).. Therefore, nonviral carrier methods have the advantage to be uniquely designed to manipulate the spatiotemporal supply of vaccine antigens and adjuvants to mucosal inductive sites. In the next sections, we describe the materials and

aspects of exclusive carrier types and provide the most recent examples of their use in the development of a mucosal vaccine (Woodrow & Kalia, 2012).

3.3.3. Nanocapsules

Emulsions and liposomes are two varieties of non-polymeric carrier systems which might be marketed for human use and have shown promise as mucosal vaccines. Nano emulsion technologies are liquid suspensions gives a long-term colloidal balance and have been used to encapsulate and give vaccines instantly onto mucosal surfaces. The dispersion of two immiscible liquids gives rise to the nanoparticles. Their size varies from 20 nm to 200 nm, which is almost similar to the size of the opportunistic pathogens, and are conveniently taken up with the aid of mucosal M cells and therefore presented to APCs (Solans et al, 2005). Table 2 presents a summary of the important classes of the nano emulsions. In brief, water-in-oil emulsions incorporate and supply hydrophilic drugs far more effectively than the oil-in-water emulsions, which are used to deliver the hydrophobic drugs (Bagwe et al, 2001). Single-nanoemulsion technology has been efficiently employed in the generation of the hepatitis B vaccine. Makidon (Makidon et al, 2008) demonstrated that recombinant HBsAg would be emulsified into uniform droplets and brought to the mucosal effector sites intranasally. The HBsAg nanoemulsion process generated a powerful immune response, producing high titers of each IgA and IgG; this robust response indicates the usefulness of nanoemulsion for NALT mucosal immunization. However, single-nanoemulsion strategies have poor controlled release profiles (Chadwicket al, 2010; Perez et al, 2001) as a result it might not be able to resist degradation inside the mucosal sites as other than NALT (Bagwe et al, 2001). Hanson (Hanson et al, 2008) presented the concept of a double-emulsion approach with a better controlled release profiles. Double-emulsion technology has been quite useful for delivering the vaccines to mucosal surfaces before they are degraded. Moreover, the double emulsions are more stable and can encapsulate the antigens without any deleterious effects to the antigen during the emulsification process (Chadwick et al, 2010; O'Hagan et al, 2001). With nano emulsion technology a novel delivery method for immunizing the mucosal immune system have been provided.

The liposomes are non-polymeric carriers that demonstrate a great promise as vaccine carriers for mucosal immunization and have been used extensively for drug delivery. The liposomes are

composed of different types of phospholipid molecules that are based on the structure of natural biological membrane lipids. The water solubility of the liposomes is very poor and they self-assemble into a phospholipid bilayer that can form a multilamellar or unilamellar vesicle that encloses an aqueous compartment (Mahato, 2005). The aqueous core of the liposome and the hydrophobic bilayer are compatible for offering lipophilic or hydrophilic cargo, respectively (Torchilin, 2005). Methods to organize the liposomes can generate small (<50-nm) or giant (>1-μm) unilamellar vesicles (Menger & Keiper, 1998; Nagayasu et al, 1999). Moreover, the selection of lipids can also be customized for distinctive function. For instance, cationic lipids may also be customized for complexation and the efficient supply of nucleic acids, and pH-titratable lipids will also be customized for pH-induced release of agents (Torchilin, 2005;Turket al, 2002; Drummondet al, 2000). Additionally, liposomes are more conveniently surface modified with ligands for tissue and the targeting, mucoadhesion, mucus penetration and steric stabilization. Although the lipid matrix has been regarded in general inert, certain lipid elements could result in infection as a result care must to be taken while selecting the suitable lipid compositions for mucosal delivery. The preparation of liposomes of varying composition, dimension, and function makes them versatile carriers for mucosal vaccines.

Many groups that utilize the liposomes for mucosal immunization against invading pathogens and viral contamination have proven promising results; which have been illustrated through various examples that is mentioned by Romero & Morilla in his recent publications (Romero & Morilla, 2011). Quite often, mucosal vaccines that utilize liposomes have been used particularly for oral or intranasal immunization. Rosada (Rosada et al, 2008) demonstrated that a single intranasal immunization with cationic liposomes delivering a DNA encoding for a tuberculosis heat-shock protein can protect against a variety of bacteria by provoking robust cell immune responses. Liposomes have also been fabricated utilizing immune modulatory lipids such as cationic LDL cholesterol derivatives or polycationic sphingolipids that can demonstrate adjuvanting activity upon mucosal supply (Guy et al, 2001; Joseph et al, 2006). Recently, an inter bilayer; pass-linked multi lamellar vesicle has been used to co-deliver the antigen and the adjuvant (Moonet al, 2011). The stabilized liposome vaccines have a greater humoral and cellular immune responses around 10–1,000-fold more compared with the responses that are caused by soluble antigen alone or by the non-cross linked multilamellar vesicles. These novel

lipid methods had been utilized as subcutaneous vaccines however it may also have an interesting activity if used for mucosal vaccines. Regardless of large number of preclinical studies that are done for checking the efficacy of liposomes as mucosal vaccines, no products have been authorized for the purpose of medical use. Future technological advancements in the future will increase our capability to better understand how the lipid matrix can be engineered to interact effectively with the mucosal immune system. (Woodrow & Kalia, 2012).

3.3.4. Nanoparticles

The flexibility of polymeric particles for mucosal vaccine design arises from the provision of distinct polymers and methods for particle synthesis, which ends up in special types of nanoparticles such as the dendrimers, micelles and solid matrix nanoparticles that are composed of synthetic or normal polymers. Usual polymer compositions of nanoparticles incorporate biodegradable or bio-eliminable synthetic polymers [such as the polyesters, polyanhydrides, poly (amino acids)] and natural polymers (for example chitosan, alginate, and albumin), copolymers, and polymer blends (Rieux et al, 2006). For drug delivery methods both synthetic and natural based micro and nanoparticle carrier are used and they exhibit best versatility for designing mighty mucosal vaccines (Rieux et al, 2006). An important purpose of nanoparticle mucosal vaccine design is to defend the antigen from degradation upon the mucosal delivery, assist to penetrate withstanding mucosal obstacles, and also to control the discharge of the antigen and co-stimulatory or immune modulatory agent in different cells and intracellular compartments. The presentations of antigen and PAMPs have primary roles in immune activation and it is governed by size and composition (surface chemistry and polymer structure) of the nanoparticle (Woodrow & Kalia, 2012).

Size performs a crucial role in the amount of antigen that may be delivered as well as the way wherein the antigen is internalized and processed via the mucosal immune system. The choice of polymers and fabrication procedure can generate nanoparticles with an extensive range of sizes and geometries. Ultra small (<25 nm) size range of polymeric micelles and dendrimers can be synthesized. Polymeric micelles are composed of amphiphilic block copolymers that normally display huge solubility differences. The polymeric micelles have both the hydrophilic and

hydrophobic segments. The solubility difference drives the assembly of nanoparticles that might be useful for delivering agents encapsulated within the core or that is attached to the polymer shell (Nagayasu et al, 1999). Despite the fact that polymer micelles are very small sizes, they are inclined to dissociate upon dilution, which is able to effect in unintended release of their cargo. Development of controlled release micellar systems have been made possible by the synthesis of new block copolymers. Dendrimers can be used for non-covalent encapsulation of vaccines or formation of covalent dendrimer-vaccine conjugates just micelles. Dendrimers offer more stability when compared with polymeric micelles as a result of the covalent bonds that type the branched polymer network (Turk et al, 2002)

Unlike polymeric micelles and dendrimers, biodegradable systems that is synthesized from natural or synthetic polymers varies in size ranging from 100 nm to >1 μm. Internalization of polymer nanoparticles is dependent on the size. As mentioned earlier, the mesh-pore spacing of mucus is 50–1800 nm (Olmsted et al, 2001), which accommodates the transport of many polymeric nanoparticle carriers as long as hydrophobic and electrostatic mucoadhesive forces is minimized. Many studies conducted explained that the most worthy size desired to strengthen the nanoparticle uptake by cells of the mucosal system. Shakweh et al. demonstrated that rhodamine 6G-labeled PLGA particles that ranged in between 0-1 μm had been internalized by the mucosal Peyer's patches, whereas bigger-sized particles were not. Conversely, NALT nanoparticle uptake recommends that the finest dimension that facilitates uptake is about 0.1 μm (Romeroet al, 2011). The kinetics of lymphatic drainage is widely influenced by the size of the nanoparticles. In most cases, nanoparticles <200 nm is easily transported by way of the draining lymph are without any problems, however the higher particles require cellular transport (Rosada et al, 2008; Guy et al, 2001). There is lot of room where more studies can be performed on the lymphatic drainage of the vaccine carrier by the mucosal tissue.

Moreover to measurement, the surface chemistry and polymer composition of nanoparticles may also be especially engineered to beat transport obstacles, have interaction with tissues and cells, and promote distinct immune-modulatory operate at mucosal sites. The hydrophobicity or hydrophilicity, as good as cost, of the nanoparticle floor can alter the microstructure of mucus and lead to mucoadhesion or mucus penetration. Hydrophobicity can also be inspiration to be a

harm-related molecular pattern that factors innate immune activation (Joseph et al, 2006). The skin of nanoparticles can be adorned with distinctive useful agencies corresponding to floor hydroxyls that bind C3b and activate complement (Guy et al, 2001). Furthermore, surface conjugation of ligands could also be priceless for introducing particularly prepared and repetitive constructions that mimic PAMPs. Jain (Jain, 2000) synthesized hydrogel nanoparticles of poly (ethylene oxide-b-propylene oxide-b-ethylene oxide), exhibiting repeating molecular motifs of protein antigens and nucleic acid danger signals. Mimicking pathogen dimension and presentation of antigen and activation indicators with nanoparticles used to be inspiration to high na¨ýve CD4+ and CD8+ T cells ex vivo more effortlessly than soluble protein antigen alone. An analogous nanoparticle design used PLGA nanoparticles to co-deliver CpG oligonucleotides and a recombinant envelope protein antigen from the West Nile virus, and it generated powerful humoral responses (Rieux et al, 2006). The worth of an identical polymeric nanoparticle designs for mucosal vaccine supply still wants to be validated (Woodrow & Kalia, 2012).

Countless polymeric nanoparticle designs have also used ligands to promote targeting and uptake through mucosal epithelia and APCs, as described above. These types of DC-targeting techniques were applied to mouse bone-marrow-derived DCs or subcutaneous DCs however now not mucosal DCs. For illustration, nanoparticles such as a PLGA core have been floor modified with a PEG lipid carrying a humanized antibody targeting DC-signal, and so they specified human DCs without difficulty (Kataoka et al, 2001). Bandyopadhyay (Bandyopadhyay& Fine, 2011) demonstrated that the density of DC-focusing on ligands on the skin of nanoparticles may just modulate the cytokine response and expression of scavenger receptors. For illustration, they tested that increased floor density of anti-DEC205 correlated with bigger move-linking of its receptor and resulted in lowered expression of CD36. This is a fundamental statement that may be useful for the design of particulate programs for vaccine delivery. Polymer-situated techniques additionally modulate intracellular trafficking to bias antigen presentation through MHC type I or MHC type II pathways. For illustration, stimuli-responsive polymers which can be sensitive to changes in pH or redox competencies had been used for intracellular focusing on of antigens. Polymer nanoparticles headquartered on propylacrylic acid and polycations have been used to advertise endosomal break out of antigens into the cellphone cytosol for MHC type I presentation. The ketal-containing or the pH-sensitive acrylic acid nanoparticles destabilize

membranes in a pH centered approach and take the advantages of the acidification of endosomes (Rajapaksa et al, 2010; Manolova et al, 2008). Polycations additionally operate to destabilize membranes through another mechanism that promotes the osmotic swelling and ultimately bursting of the endolysosomes due to the sequestration of protons by utilizing the biomaterial (Reddy et al, 2007). Redox-touchy polymers have moreover been mostly explored to take the advantage of the redox potentials within the endosome (reductive) versus the lysosomes (oxidative) (Seong& Matzinger, 2004). The redox-sensitive polymers are usually stimuli-responsive polymers and in the future they will be a valuable element for the development of mucosal vaccine.

Particulate delivery system offers opportunities to control the timing of antigens and adjuvant presentation. Emulsification methods are used regularly to produce nanoparticles. Nanoparticles can also be formed from emulsions by means of number of different techniques that cause the polymer precipitation upon the solvent removal by means of extraction, diffusion, de-salting or evaporation (Moon et al, 2011) The gelation of polymers that are dispersed in emulsion droplets can also produce nanoparticles (Dementoet al, 2010). This strategy is crucial only to those polymers that exhibit gelling properties in different environmental situation such as temperature, pH, or addition of cross linking agents. Dispersion of monomers within emulsion droplets can produce the nanoparticles through in situ polymerization. Particulate carriers that are fabricated utilizing the co-polymer PLGA represents one of the most widely techniques for the delivery of vaccines. PLGA has a first-class controlled-release profile, one of the best toxicological profiles, and US FDA (Food and Drug administration) approval (Moonet al, 2011). Woodrow et al. (Woodrow et al, 2009) demonstrated that the topical delivery of fluorescently labeled PLGA nanoparticles to the vaginal mucosa resulted in distribution and penetration of the particles throughout the regional tissues. The tissue distribution is a result of cellular uptake and trafficking. The surfaces of PLGA nanoparticles can be easily modified to enhance the physicochemical properties so that the particle is easily diffused through the mucosa and transcytosis via using mucosal M cells (Neutra& Kowlozski, 2006; Moon et al, 2011, Bandhopadhaya & Fine, 2011). One of the main advantages of the PLGA is that vaccine antigens can be encapsulated into the matrix or on the surface of PLGA carriers (Bandhopadhaya & Fine, 2011). Although the PLGA nanoparticles had been widely used for subcutaneous or

intramuscular depot injections, people are trying to study these nanoparticles so that they can be effectively utilized for the mucosal immunization (Flanaryet al, 2009). Efforts have been taken to describe the optimal condition in order to improve the uptake of the PLGA nanoparticles across the mucosal barrier (Woodrow & Kalia, 2012).

3.4. Mucosal Adjuvants

In order to enhance the immune function vaccination through the mucosal route requires the use of a potent adjuvant. Nevertheless, few mucosal adjuvants exhibit ample potency without being poisonous or reactogenic, and however amongst these only few are accepted for human use (Table 2). The aluminum salts and specific forms of emulsions are the only adjuvants that are permitted in the United States. An injectable vaccine for hepatitis B and HPV (Cohen et al, 2008) was recently approved in the United States. The vaccines were composed of Aluminium salt with TLR4, which is also known as monophosphoryl lipid A or MPL (Ofek et al. 2010). The mechanism of action of adjuvants is poorly understood, and as result of this poor understanding the development and rational design of new adjuvant compounds have been hampered. Considering that the approval of aluminum salts as an adjuvant practically eighty years ago, its mechanism of actions is still controversial. The procedures utilized in immune-potentiation of mucosal vaccines have been highlighted and the up to date advances that can enhance the development of novel mucosal adjuvants have also been discussed.

The immune-potentiating activity of the adjuvants could also be mediated by means of special mechanisms of action (Table 2). Adjuvants can increase the immunogenicity of vaccine antigens by using producing a pro-inflammatory atmosphere that recruits and promotes the infiltration of phagocytic cells— particularly APCs—to the site of injection. By means of enhancing antigen presentation, by way of activating APCs or by inducing cytokine expression the adjuvants can exert their immunological effect. The arrangement of a vaccine antigen and a targeted adjuvant continues to be mostly an empirical approach; nonetheless a number of adjuvants which were evaluated in the scientific or clinical trial are recognized to result in specific effector adaptive immune responses. Of particular concern, the mucosal adjuvants are usually toxin-based

adjuvants [cholera enterotoxin (CT), heat-labile enterotoxin (LT),], immune stimulatory adjuvants (e.G., QS21, CpG, MPL,), and particulate adjuvants [e.G., immune stimulating complexes (ISCOM), emulsions,] (Woodrow & Kalia, 2012).

Probably the strongest mucosal adjuvants that are available are the LT from *E. Coli* and CT from *V. Cholerae*, but they are too poisonous for being used in humans. As a consequence, mutants of the native toxins had been generated with the aid of site-directed mutagenesis that decreases the enzymatic exercise of CT and LT. The mutants of LT are LTK63 and the LTK72 which are inactive in nature and have tremendously lowered ADP-ribosylating undertaking, respectively. LT mutants had been evaluated as adjuvants for variety of mucosal immunization routes that includes the oral, nasal, and anogenital routes. Intranasal immunization with LT mutant adjuvants and proper vaccine antigens has led to protection against the HSV (Xiaet al, 2008)*Streptococcus pneumoniae* (Cohenet al, 2008) and the *Bordetella pertussis* (Hubebell et al, 2009). LT mutants have additionally been used to adjuvant HIV-1 p55 gag subunit vaccines that are administered orally and intranasally (Campos et al, 2001). In these examples, the mechanism of protection used to be principally as a result of induction of strong cytotoxic T lymphocyte responses. The mechanism of motion of CT and LT is concept to come back up from increased permeation of antigens across epithelial boundaries and a marked enhancement in the antigen presentation with the aid of APCs (Woodrow et al, 2009; Jung et al, 2000). Oral vaccines that are adjuvanted with CT or LT was implicated in the M cells which essential for the antigen sampling and the uptake in intestinal sites (Manicassamy& Pulendran, 2009).

As discussed earlier, the main target of the immunologist is the innate immune receptors for the adjuvant activity and in some circumstances TLR ligands had been deliberately integrated into experimental mucosal formulations. For example, bacterial flagellin, which is a ligand for each and every TLR5 and the NLR IPAF/NLRC4, reveals adjuvant activity in mucosal immunization studies. Synthetic ligands for TLR9 (for example the CpG) are still undergoing the clinical trials to produce different mucosal immune responses to allergens. There is the necessity of more studies to be conducted in this area, as the aforementioned bacterial toxins have been identified to produce the adjuvant activity (for example the CT, LT) but it still needs to be connected to specific immune response. This would suggest that there is further complexity in immune

adjuvant responses that are neutral of innate immune signaling pathways, or that the crucial pathways have not been recognized. Additionally, regulation of the mucosal immunity and the anatomy of the mucosal tissue would additionally require specific mucosal adjuvants that are not connected to the systemic immune responses (Woodrow & Kalia, 2012).

Finally it is mentionable that while developing the vaccine carrier system the concept of the addition of adjuvant was not present. A variety of polymers including PLGA and chitosan had been utilized in plenty of vaccine formulations in order to give their effect on the antigen release and its adhesion to the mucosal surfaces. However these polymers have not undergone any test to prove their ability to promote innate immune response. The fundamental aspect of a powerful vaccine includes its ability to stimulate the immune system, although in some occasions the polymeric nanoparticles is able give an immune stimulating activity. Chitosan-based nanoparticles are not only a strong mucoadhesive but they also enhance the absorption via the mucosa lining considering their intrinsic capability to open tight junctions (Alpar et al, 2005). Read (Read et al, 2005) demonstrated that the chitosan particles that had been dropped at the mucosal tissues had been immuno-stimulatory, producing cytokines corresponding to the interferon γ, IgG and the sIgA (Laurent et al, 2009). Baaten (Baaten et al, 2010) have recently demonstrated that the chitin-based nanoparticles can broaden innate immunity when delivered to the NALT with the help of up regulation of cytokines tumor necrosis part α, IL-6 post immunization and the interferon γ. These examples gives us a view that the polymers could be modified to provide specific immune response such as the TLR2 and the TLR5 , thus assuming that these modified polymers can help in restoring the physical characteristics and hence could be used as the carriers of vaccines. Thus concluding, we can say that there exists many opportunities for further development in this field and if provided, the quick advances in immunology, extra rational approaches for adjuvant development should not be impossible (Fraseret al, 2007).

Mucosal Adjuvant	Mucosal route	Proposed mechanism of action	Antibody	T cells	Cytokines and chemokines
Heat-labile enterotoxin	Oral, nasal, vaginal, rectal	Enhancement of antigen presentation by APCs	IgG1, IgG2, IgA	Th1/Th2, CD8	IL-6, IL-8, IL-10, IL-1(α,β)
Cholera toxin	Oral, nasal, vaginal, rectal	Enhancement of antigen presentation by APCs	IgA, IgG1, IgE	Th2, CD8	IL-4, IL-5, IL-6, IL-10
MPL	Oral, nasal, vaginal, rectal	TLR4		Th1/ Th2	IL-1, IL-17, IFN- γ
CpG	Oral, nasal, vaginal, rectal	TLR9	IgG2, IgA	Th1/Th2, CD8	IL-6, IL-12, IL-8, RANTES, MIP-1α, MIP-1β, TNF- α, IFN- γ
Protollin (LPS)	Nasal	TLR4, TLR2	Serum IgG, IgA	Th1/ Th2	MIP-3, IFN- γ, IL-18
Flagellin	Nasal	TLR5, TLR4	IgG , IgA	Th1/ Th2	MIP-2, TNF-α, IFN- γ, IL-6
QS21	Oral, nasal		IgG1, IgG2a, IgG2b, IgE	Th1	IL-4, IL-5, IL-6, IL-10
Chitosan	Oral, nasal	Electrostatic interaction with mucus	Serum IgG, sIgA	Antibody dependent response	IL-1β , IL-18

		and cell surfaces			
PLGA microparticles	Oral, nasal, vaginal	Mechanism unknown	Antigen dependent antibody	Antibody dependent response	Depends on immunostimulation
Emulsions	Oral, nasal	Mechanism unknown	Antigen dependent antibody	Th1/ Th2	Depends on immunostimulation
\Liposomes	Oral, nasal	Application specific mucosal IgA and IgG	Antigen specific mucosal IgA and IgG	Antibody dependent response	IL-2 proliferation
ISCOM	Oral, nasal , vaginal	IgG1, IgG2a	IgG1, IgG2	Th1/Th2, CD8	Not determined
Virus-like particles	Oral, nasal, vaginal	Both Serum IgA and IgG	Both serum IgG and IgA	Th1/ Th2	Not determined

TABLE 3: Immunological considerations of known mucosal adjuvants (adapted from Woodrow *et al*, 2012)

CONCLUSION

Mucosal vaccination is a needle and medical waste free vaccines 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 inclined to degradation requiring a mighty delivery procedure and on the whole they give low immunogenicity. 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 carrier programs can restrict degradation or premature neutralization by utilizing the immune systems components. On this approach particle-mediated delivery systems may additionally develop the potential vaccine administration routes which could prove to be vital in successful delivery of the vaccines to distinct tissues. They are able to be generated from quite a lot of substances, like the polymers, lipids and metals, to be in a narrow size range with exact surface traits. For controlled release, layered systems can be designed and the targeting moieties can additionally be incorporated to strengthen the delivery. Particle-mediated delivery programs have the ability to effectively deliver DNA vaccines, which can be then modified to improve by incorporation of extra antigenic or adjuvant components that is encoded within the DNA or integrated in or onto the particle delivery process. The usage of particle-mediated supply programs is an effective method to enhance mucosal vaccination by defending the immunogenic materials during the delivery process, providing specific delivery methods, and also allowing the integration of adjuvant materials.

FUTURE WORK

Although the live-attenuated pathogens can result in an effective protective immunity but it is still is not full known which of the innate and adaptive immune pathways make the most contribution to the induction of specific responses such as the sIgA. Building on this knowledge shall be principal in development for targeting specific responses. With the introduction of new approaches for target delivery, the vaccine antigen delivery formulations are also benefitted. Nevertheless, in lots of cases, the mucosal targeting is directed best toward the mucosal epithelium, without consideration of the certain contribution of M cell–mediated immune surveillance or the properties of the epithelia such as the vaginal mucosae. Gathering information about every aspect would be beneficial in the development of formulations which are tailored for specific targets (Woodrow & Kalia, 2012). A continuous source of adjuvant trigger and antigens is provided by the live pathogenic microbes but there is very little knowledge about the optimal timing for the release of the antigen and the immune cell stimulation but the engineered controlled release formulations must have a target (Woodrow & Kalia, 2012).. Within the initial induction of immune responses, the contributions of adjuvant ligands (for example TLRs and NLRs) in stimulating innate immune pathways are well founded in traditional immunology but much less studied in the mucosal immune method. Some vaccine antigens are more immunogenic hence there is need for the adjuvants. Many mucosal vaccine formulations have blended adjuvant ligands (for example the lipopolysaccharides, CpG) with antigens, but extra cautious designs and the new routes of vaccine uptake can be considered to bring about effective results of adjuvants on more cell types and not only the DC (Woodrow & Kalia, 2012)..

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