2.1 Immune System: In Brief: The immune system is an extraordinarily adaptive defense system that has evolved to protect vertebrates from invading pathogenic microorganisms and cancer. A plethora of cells and molecules are secreted in response to any invading agent in order to eliminate it. Broadly, an immune response can be bisected into two interrelated activities—recognition and response. Immune recognition exhibits outstanding specificity. The immune system is capable of distinguishing minor chemical differences that discriminate and differentiate one foreign pathogen from another. Furthermore, the system also differentiates between foreign molecules and self-cells and proteins. After recognition of any foreign invader, the immune system join up variety of cells and molecules to mount an appropriate response, known as an effector function intended for elimination or neutralization of the invader. Future exposure to the same foreign organism induces a memory response, characterized by a heightened immune reactivity that assists in early and fast elimination of pathogen and prevention of disease.

Immunity owns both non-specific and specific components. Innate or non-specific immunity refers to the basic resistance to disease. Innate immunity can be envisioned as comprising four types of defensive barriers, anatomical, physiological, endocytic/phagocytic, and inflammatory. Physical or anatomical barriers tend to prevent the entry of pathogens being first line of defense against infection. The skin and the surface of mucous membranes are included in this category because they provide an effective barrier to the entry of most microorganisms. Physiologic barriers comprise temperature, pH, oxygen tension and various soluble factors. Soluble factors that contribute to non-specific immunity include soluble proteins such as lysozyme, interferon and complement. Another noteworthy innate defense mechanism is the ingestion of extracellular macromolecules and particles through endocytosis and phagocytosis, respectively. In endocytosis, the macromolecules contained within the extracellular tissue fluid are internalized by cells. Endocytosis occurs through one of two processes: pinocytosis or receptor-mediated endocytosis. Phagocytosis involves the ingestion of particulate material, including whole pathogenic microorganisms. In phagocytosis, which differs from endocytosis in several ways, the plasma membrane expands around the particulate material to form large vesicles called phagosomes. Once particulate material is ingested into phagosomes, the phagosomes fuse with lysosomes and the ingested...
Characterization of HPV candidate antigen and Development of novel vaccine delivery system

Chapter 2: Review of Literature

11

material is then digested in the endocytic processing pathway by a process similar to that observed in endocytosis. Inflammatory response, which is a complex sequence of events induced by tissue damage caused by a wound or by invasion by a pathogenic microorganism. Inflammatory response also contributes to non-specific defense against the invading pathogen. Three major events that occur during an inflammatory response are vasodilatation, increased capillary permeability and influx of phagocytic cells (Kuby, 1994).

Acquired or specific immunity represents functional arm of immune system capable of specifically recognizing and selectively eliminating or neutralizing foreign microorganisms and molecules. In contrast to innate immunity acquired immunity displays specificity, diversity, memory and also self/non-self-recognition. The specificity of the immune system can be reflected from its capacity to distinguish minor differences among antigens. Most of the antigens are macromolecules either proteins or large polysaccharides. The immune system is capable of generating tremendous diversity in its recognition molecules, allowing it to specifically recognize billions of uniquely different structures on foreign antigens. Once the immune system has responded to an antigen, it exhibits memory. Future exposure to the same antigen, it induces an exaggerated immune response. Moreover, the ability of immune system to respond only to foreign antigens indicates that the immune system is capable of distinguishing self from non-self. It is essential that the immune response be limited to non-self-antigens since inappropriate response to self-antigens can lead to autoimmune disorders.

2.2. The Cells of the Immune System: Two major groups of cells, lymphocytes and antigen presenting cells are involved in elicitation of an effective immune response. Lymphocytes are white blood cells (WBCs) produced in the bone marrow, circulate in the blood and lymph system and finally reside in various lymphoid organs. The attributes of specificity, diversity, memory and the self/non-self-recognition are mediated by the lymphocytes. Antigens are recognized by lymphocytes by means of membrane receptors specific for the foreign material. Two major populations of lymphocytes that exist are B lymphocytes and T lymphocytes (Kuby, 1994).

2.2.1 B Lymphocytes: The B lymphocytes derive the name from its site of maturation in the bursa of Fabricius in birds; the name turned out to be apt, for its major site of maturation in mammals in the bone marrow. Mature B cells can be distinguished from other lymphocytes
by the presence of membrane bound immunoglobulin (antibody) molecules, which serve as receptors for antigen. When a naïve B cell first encounters the antigen for which its membrane bound antibody is specific, the cell begins to divide rapidly; its progeny differentiate into memory B cells and effector cells called plasma cells. Memory B cells have longer life span and continues to express membrane bound antibody with the same specificity as the original parent cell. Plasma cells do not produce membrane bound antibody but instead produce the antibody in original parent cell. Plasma cells live only a few days but they secrete enormous amounts of antibodies (~2000 molecules/sec) during this time. These secreted antibody molecules serve as the major effector molecule of humoral immunity.

2.2.2 T Lymphocytes: T lymphocytes derive their name from their site of maturation in the thymus. Like B lymphocytes, these cells have membrane receptors for antigen. The T cell receptor molecule is structurally a heterodimer composed of two protein chains, either alpha and beta or gamma and delta which are linked by disulfide bonds. The amino-terminal ends of the two chains fold together to form the antigen binding cleft of the T cell receptor. Unlike membrane bound antibodies on B cells, which can recognize antigen alone, T cell receptors can recognize antigen only in association with cell membrane proteins known as major histocompatibility complex (MHC) molecules. This points to fundamental difference between humoral and cell mediated branches of the immune system. Whereas B cell is capable of binding soluble antigen, the T cell system is restricted to binding antigen displayed on self-cells.

This antigen may be displayed together with MHC molecules on the surface of antigen presenting cells or on virus-infected cells, cancer cells and grafts. The T cell system has developed to eliminate these altered self-cells, which pose a threat to the normal functioning of the body. There are two subpopulations of T cells: T helper (T\(_{H}\)) cells and T cytotoxic (T\(_{C}\)) cells. Presence of third type of T cells, called T suppressor (T\(_{S}\)) cells is doubtful. The T\(_{H}\) and T\(_{C}\) cells can be distinguished by their display of one of the two membrane glycoproteins, either CD4 or CD8. T cells displaying CD4 generally function as T\(_{H}\) cells and those with CD8 as T\(_{C}\) cells. In response to the recognition of an antigen MHC complex, T\(_{H}\) cells secrete various growth factors collectively known as cytokines. As a T\(_{H}\) cell is activated it becomes an effector cell secreting various cytokines, which play an important role in activating B
cells, T\textsubscript{C} cells, macrophages and various other cells that participate in the immune response. Differences in the pattern of cytokines produced by activated T\textsubscript{H} cells results in qualitative differences in the type of immune response that develops. Under the influence of T\textsubscript{H} derived cytokines, a T\textsubscript{H} cell that recognizes an antigen-MHC molecule complex proliferates and differentiates into an effector cell called a cytotoxic T lymphocyte (CTL). In contrast to the T\textsubscript{H} cell, the CTL generally does not secrete many cytokines and instead exhibits cytotoxic activity. The CTL has a vital function in monitoring the cells of the body and eliminating those that display antigen, such as virus, infected cells, tumor cells and cells of a foreign tissue graft.

2.2.3 Antigen presenting cells and processing pathways: Cells that are able to present peptides to T cells are referred to as antigen presenting cells (APCs). Since cells expressing either class I or class II MHC molecules can present peptides to T cells, technically they could be classified as antigen presenting cells. However, by convention, those cells that display peptides associated with class I MHC molecules to CD8\textsuperscript{+} T\textsubscript{C} cells are referred to as target cells, only those cells that display peptides associated with class II MHC molecules to CD4\textsuperscript{+} T\textsubscript{H} cells are called APCs. A variety of cells can function as APCs. The distinguishing feature of these cells is their ability to constitutively express class II MHC molecules. These cells internalize exogenous antigen either by phagocytosis or by endocytosis, process it within the endocytic pathway and display the resulting antigenic peptides together with class II MHC molecules on their membrane.

Among the cells that constitutively express class II MHC molecules and function as APCs are macrophages, B cells, dendritic cells, and Langerhans cells, thymic dendritic and epithelial cells. Several other types of cells also can be induced to express class II MHC molecules during a sustained inflammatory response and thus can function as APCs for short periods of time. These include thyroid epithelial cells, glial cells, pancreatic beta cells, skin fibroblasts and vascular endothelial cells. Since nearly all nucleated cells express class I MHC molecules, virtually any nucleated cell is able to function as a target cell capable of presenting endogenous antigens to T\textsubscript{C} cells. Most often target cells are those cells that have been infected by a virus or some other intracellular microorganism. However, target cells can also be cells from a graft, altered self-cells such as cancer cells or ageing body cells.
Intracellular or endogenous and extracellular or exogenous antigens present different challenges to the immune system. Extracellular antigens are eliminated by secreted antibody, whereas intracellular antigens are most effectively eliminated by CTLs. To mediate these responses, the immune system uses two different antigen-presenting pathways: exogenous antigens are processed in the endocytic pathway and presented on the membrane with class II MHC molecules, and endogenous antigens are processed in the cytosolic pathway and presented on the membrane with class I MHC molecules (Figure 1).

**Figure 1:** Overview of the humoral (MHC II) and cell-mediated (MHC I) branches of the immune system (Kuby, 1994)
2.3 Organs of the Immune System: A number of morphologically and functionally diverse organs contribute to generation of immune response. These organs can be divided into the primary and secondary lymphoid organs on the basis of their function. Immature lymphocytes generated during hematopoiesis mature and become committed to a particular antigenic specificity within the primary lymphoid organs. Only after a lymphocyte has matured within a primary lymphoid organ it is capable of mounting an immune response. In mammals, the primary lymphoid organs are the bone marrow, where B lymphocytes mature and the thymus where T lymphocytes mature. A variety of secondary lymphoid organs exist each uniquely suited to trap antigen from defined tissues or vascular spaces and to provide sites, where mature, immunocompetent lymphocytes can interact effectively with that antigen. The lymph nodes collect antigen from the intracellular tissue fluids, whereas the spleen traps blood-borne antigens. The respiratory and gastrointestinal tracts possess aggregations of mucosal associated lymphoid tissue (MALT) including Peyer’s patches, tonsils, adenoids and the appendix which are capable of trapping antigens entering through different mucosal surfaces.

2.4 Antibodies: Immunoglobulins function as antibodies, the antigen binding proteins that are present on the B cell membrane and also are secreted by plasma cells. Secreted antibodies circulate in the blood and serve as the effectors of humoral immunity by searching out the neutralizing or eliminating antigens. All immunoglobulins share certain structural feature (Figure 2), bind to antigen and participate in a limited number of effector functions (Table 2a). Membrane bound antibody confers antigenic specificity on B cells; antigen specific proliferation of B cell clones depends on interaction of membrane antibody and antigen.

Figure 2: Structure of Immunoglobulin (Kuby, 1994)
Table 2a: Different classes of immunoglobulins

<table>
<thead>
<tr>
<th>Properties</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
<th>IgD</th>
<th>IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td>Monomer</td>
<td>Pentamer</td>
<td>Dimer (with secretory component)</td>
<td>Monomer</td>
<td>Monomer</td>
</tr>
<tr>
<td>% of total serum antibody</td>
<td>80</td>
<td>5-10</td>
<td>10-15 (in serum)</td>
<td>0.2</td>
<td>0.002</td>
</tr>
<tr>
<td>Location</td>
<td>Blood, lymph, intestine</td>
<td>Blood, lymph, B cell surface (as monomer)</td>
<td>Secretions (tears, milk, saliva, mucus, intestine), blood, lymph</td>
<td>Bcell surface, blood, lymph</td>
<td>Bound to mast and basophil cell throughout body, blood</td>
</tr>
<tr>
<td>Mol. Weight</td>
<td>150,000</td>
<td>970,000</td>
<td>405,000</td>
<td>175,000</td>
<td>190,000</td>
</tr>
<tr>
<td>Half-life in serum</td>
<td>23 days</td>
<td>5 days</td>
<td>6 days</td>
<td>3 days</td>
<td>2 days</td>
</tr>
<tr>
<td>Complement fixation</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Placental transfer</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Known functions</td>
<td>Enhances phagocytosis, neutralizes toxins and viruses, protects fetus and newborn</td>
<td>Especially effective against micro-organisms and agglutinating antigens, first antibodies produced in response to initial infection</td>
<td>Localized protection on mucosal surfaces</td>
<td>Serum function not known, presence of B cells functions in initiation of immune response</td>
<td>Allergic reactions, possible lysis of parasitic worms</td>
</tr>
</tbody>
</table>

2.5 Aluminium adjuvants: The use of aluminium compounds as adjuvants was first described by Glenny more than 70 years ago. This was the first of many experiments demonstrating that aluminium salts, especially aluminium hydroxide and aluminium phosphate, possess adjuvant activity (Gupta and Relyveld, 1993). Aluminium hydroxide (Al(OH)₃) and its salts have been successfully utilised for many years in inactivated (e.g. Diphtheria, Tetanus) and recombinant vaccines (e.g. Hepatitis B) where protection is dependent on the generation of neutralising antibodies. This has generally been in the form of vaccines adsorbed to preformed aluminium hydroxide or aluminium phosphate hydrated gels.
(Alum) in order to overcome problems with the heterogeneity of protein aluminate precipitates.

Adsorption of antigens on aluminum adjuvants depends upon physical and chemical characteristics of antigen, type of aluminum adjuvant and conditions of adsorption. The physico-chemical characteristics are very useful in optimizing the adsorption of antigens onto aluminum adjuvants and depend on the nature of the individual antigen.

The two most important mechanisms of antigen adsorption by aluminum-containing adjuvants are electrostatic attraction and ligand exchange. Adsorption of antigens on aluminum salts depends heavily on electrostatic forces between adjuvant and antigen and other interactions include hydrophobic, Vander Waals and hydrogen bonding which contribute to the adsorption of antigens on aluminum adjuvants. However, these forces may not suffice to cause adsorption of antigen if the same charge or electrostatic repulsive force is present on antigen and the adjuvant. The electrostatic attraction is a weak charge dependent interaction force between the antigen & adjuvant and the most frequently encountered adsorption mechanism where as in ligand exchange the phosphate group of a phosphorylated antigen exchanges with a surface hydroxyl of the adjuvant resulting in a stronger adsorption force.

Unlike electrostatic adsorption, ligand exchange by phosphate is not affected by the relationship between the isoelectric point (IEP) of the antigen or the aluminum-containing adjuvant and the pH of the vaccine. Ligand exchange is also not sensitive to ionic strength as is electrostatic adsorption. In addition, other intermolecular binding forces like hydrophilic, hydrophobic, Vander Waals interactions, may play a role in protein adsorption and every binding force has its influence in a given antigen–adjuvant combination depending on the nature of the antigen and the chemical environment (pH, ionic strength, presence of surfactants etc.).

2.5.1 Factors affecting antigen adsorption onto aluminum adjuvant

The factors affecting the antigen adsorption on the aluminum adjuvant may be classified into two categories:  i) Adjuvant dependent factors ii) Antigen dependent factors
i) **Adjuvant dependent factors:** The two most commonly used aluminum adjuvants, aluminum hydroxide and aluminum phosphate, have different points of zero charge. At neutral pH these gels have opposite charges, wherein aluminum phosphate is negatively charged and aluminum hydroxide is positively charged. It is important to select the aluminum adjuvant carefully on the basis of the charge of the antigen at neutral pH. Other physical conditions affecting adsorption of antigens on aluminum adjuvants include pH, temperature, size of the gel particles and ionic strength of the formulation.

ii) **Antigen dependent factors:** As a general guideline, for many protein antigens adsorption is best accomplished in the pH interval between the isoelectric point (IEP) of the protein antigen and the point of zero charge (PZC) of the aluminum adjuvant. This applies to both aluminum hydroxide and aluminum phosphate adjuvants. In this interval the adjuvant and the antigen will have opposite electrical charges, facilitating electrostatic attraction and adsorption. It has been noticed that aluminum hydroxide is superior to aluminum phosphate in adsorbing proteins with an acidic IEP and vice versa for proteins with an alkaline IEP. However, if the antigen contains phosphorylated groups, ligand exchange between the antigen-associated phosphate and hydroxyl groups of the adjuvant may account for a high affinity binding to the adjuvant. The primary mechanisms responsible for adsorption have been explained, in part, by electrostatic attraction and, in part, by anionic ligand exchange. In addition, other intermolecular binding forces, including hydrophilic–hydrophobic interaction, may play a role in protein adsorption and each binding force may play its role in a given antigen–adjuvant combination depending on the nature of the antigen and the chemical environment (pH, ionic strength, presence of surfactants etc.).

However, although Alum is particularly potent at inducing such humoral immune responses, it is a poor stimulator of classical cell mediated immunity (CMI). Control of cellular and humoral immune responses has been attributed to mutually antagonistic subsets of CD4+ T helper (Th) lymphocytes. Activation of the Th1 subset is associated with the production of Interleukin-2 (IL-2) and Interferon-g (IFNg) which induces B cell production of the IgG2a subclass of antibodies and results in the development of a classical cell mediated immune response (Mosmann and Cherwinski, 1986; Cher and Mosmann 1987). Conversely, activation of the Th2 subset and the subsequent production of cytokines such as...
IL-4, IL-5, IL-6 and IL-10, induces B cell IgG1 and IgE production and is associated exclusively with the development of classical humoral immune responses (Boom and Liano, 1988; Mosmann and Coffman 1989). Also for many reasons as explained in the introduction section, the development of new and more effective vaccine adjuvants is a key immunological objective which requires resolution before the full impact of new generation vaccines can be realised.

2.6 Mucosal Immune System: The mucous membranes lining the digestive, respiratory and urogenital system, which have a combined surface area of about 400 m², are the major sites of entry for most pathogens. To guard against such infections, these surfaces are protected by a complex system of defenses that comprise the mucosal immune system. This protection is provided by organized lymphoid tissues known collectively as mucosal associated lymphoid tissue (MALT). The functional importance of MALT in the body’s defense is attested by its large population of antibody producing plasma cells in the spleen, lymph nodes and bone marrow combined. Various inductive sites for mucosal immunity include nasal associated lymphoid tissues (NALT), gut associated lymphoid tissues (GALT), genital tract, salivary glands, ocular tissues and mammary glands collectively known as common mucosal immune system (Figure 3). These sites are in intimate and constant contact with the external environment, contain mucocilliary epithelium, possess secretory component and/or sIgA in the epithelium and lamina propria, and contain organized lymphoid follicles in subepithelial regions. These tissues participate in circulation of antigen reactive IgA, B lymphocytes and specifically sensitized T cells to other distant sites, after stimulation in the MALT (McGhee et al., 1992; Shalaby, 1995).

2.6.1 The Common Mucosal Immune System (CMIS): Communication between the MALT and distant mucosal surfaces through cell trafficking has been termed the ‘common mucosal immune system’. With regard to the BALT, this would seem to be predominantly a case of gut to bronchus movement of cells. It has been suggested that, in view of a relative paucity of immunocompetent tissue in the BALT, a priming of the intestine followed by a booster exposure of antigen in the respiratory tissue could be more effective in inducing mucosal immune responses than immunization of the respiratory tract alone (Pabst, 1992). However, this suggestion has not been borne out by practice. As discussed above, it has been
shown by various workers that nasal administration (IN) of antigen can result in a better level of IgA in the intestine than oral administration. Such an effect may well be due to a difficulty in delivering suitably large quantities of antigen to the correct region(s) of the gut due to dilution effects and to the degradation of sensitive antigenic structures in the acid environment of the stomach. Nasal and (to a lesser extent) pulmonary administration of antigen is an efficient process where it should be possible to administer a dose of vaccine to a preferred site.

A model for the homing of primed lymphoid cells from mucosa-associated lymphoid tissue (MALT) with its activated lymphoid follicles, to effector sites in the integrated human mucosal immune system is shown in Figure 1.4 (Brandtzaeg et al., 1999). The components of common mucosal immune system in human are expressed in Table 1.4 (Ogra et al., 2001). Putative regionalization in communication between inductive and effector sites is indicated, the heavier arrows representing preferential B-cell migration pathways. Homing from gut associated lymphoid tissue (GALT) is believed to be determined mainly by an integrin on primed cells, interacting with a mucosal addressin cell adhesion molecule expressed on the micro vascular endothelium in the intestinal lamina propria. Other adhesion molecules appear to be employed by immune cells primed in bronchus-associated lymphoid tissue (BALT) and nasal-associated lymphoid tissue (NALT). Brandtzaeg et al., (1999) suggest that the urogenital tract might employ similar molecular homing mechanisms as those of the upper respiratory and digestive tracts and therefore appears to receive primed immune cells from the NALT. Lactating mammary glands also appear to receive primed cells from both types of inductive tissue (Davis, 2001).
2.7 Mucosal Immunization: Mucosal administration involves delivery by nasal, oral, pulmonary and urinogenital routes. Delivery of an antigenic substance via these routes is known as mucosal vaccination. Mucosal vaccination has merits over parenteral vaccination that makes it a good alternative to conventional parenteral administration. It provides dual immunity - mucosal as well as systemic (Holmgren, et al., 2003 & Davis, 2001). Parenteral vaccination results in production of IgG antibodies that give protection against a pathogen in systemic circulation only. Since IgG have no role in mucosal immunity, pathogens can invade the body through this route. On the contrary, mucosal delivery results in production of IgA (also called as secretory antibodies) as well as IgG. Since a pathogen has more than one portal of entry, most of them use one or more mucosal routes; it is thus the best way to trap the pathogen at its entry level. This is done by the surface IgA (sIgA) antibodies (Vyas, et al., 2007 & Van der Lubben, et al., 2001).

Those pathogens invading through the parenteral route are acted upon by the IgG, present in systemic circulation. Another merit associated with mucosal vaccination is that, an immune response at one site also leads to the development of immunity at a distant site. This
occurs due to the interconnected immunological network of sIgA, also called common mucosal immune system (CMIS). Broadly, CMIS consists of gut associated lymphoid tissues (GALT), bronchus associated lymphoid tissues (BALT), nasal associated lymphoid tissues (NALT) and larynx associated lymphoid tissues (LALT) (Mestecky, *et. al.*, 1997 & Neutra, *et.al.*, 2006). Parenteral methods of vaccination are always uneconomical due to the strict adherence to sterility and the need of trained medical personnel for administration. They are often associated with needle borne infections like Human Immunodeficiency Virus (HIV) or Hepatitis B due to contaminated needles, especially in developing countries like India. Pain and trauma leads to development of phobia, especially in children. All these problems can be circumvented by mucosal vaccination.

I. It induces the production of IgA antibody that prevents the non-invasive pathogen (e.g. non bacteria) from attaching and colonizing at mucosal surface.

II. Prevents invasive pathogen (e.g. virus and invasive bacteria) from penetrating and replicating in mucosal cell (Hobson, *et al.*, 2003).

III. Prevents attachment of bacterial toxin to the mucosal surface.

IV. IgG production prevents the attachment and penetration of pathogen that gain access through the systemic circulation into various tissues.

V. Activate cell mediated immune response which helps in eliminating intracellular pathogen (Czerkinsky, *et al.*, 1999). Mucosal vaccination offers protection against infections by one of the following mechanisms;

**2.8 Various Routes of Transmucosal Vaccination:** There are seven routes for mucosal vaccination namely oral, nasal, sublingual, pulmonary, rectal, vaginal and ocular route. Table 2b illustrates comparative overview of different mucosal routes.
Table 2b: Components of common mucosal immune system in humans (Ogra et al. 2001)

<table>
<thead>
<tr>
<th>Inductive sites</th>
<th>Effector sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organized lymphoid follicles in:</td>
<td>Lymphoid cells in lamina in lamina propria of:</td>
</tr>
<tr>
<td>• NALT</td>
<td>• Salivary glands</td>
</tr>
<tr>
<td>• BALT</td>
<td>• Occular tissues</td>
</tr>
<tr>
<td>• GALT</td>
<td>• Conjunctiva</td>
</tr>
<tr>
<td>• Appendix</td>
<td>• Bronchial mucosa</td>
</tr>
<tr>
<td>• Rectal lymphoepithelial tissue</td>
<td>• Gastrointestinal mucosa</td>
</tr>
<tr>
<td>Peritonal B cells?</td>
<td>• Urogenital tract</td>
</tr>
<tr>
<td></td>
<td>• Mammary gland</td>
</tr>
<tr>
<td></td>
<td>Middle ear mucosa</td>
</tr>
</tbody>
</table>

2.8.1 Oral Route: This is the most simple and hence an attractive route for vaccination. Administration of vaccines orally generates immune response in small intestine, ascending colon, and distal tissues like mammary and salivary glands through CMIS. But it fails to induce immune response in tonsils and the female genital track (Hobson, et al., 2003). Gut associated lymphoid tissues (GALT) is responsible for the production of sIgA antibodies. GALT consist of primarily the peyers patches which act as a major inductive site for humoral as well as cell mediated immunity (Czerkinsky, et al., 1999). It has been observed that particulate antigens show a greater response as compared to soluble antigen. This is because of their better uptake by peyers patches. Although an attractive means of administration, oral route has some limitations associated with it. Degradation of antigens in stomach because of strong acidic environment and enzymatic activity are commonly associated with the oral route. Means of overcoming this includes encapsulating the antigen in a suitable carrier system, protecting it from a damaging environment.

2.8.2 Nasal Route: Nasal mucosa presents a good alternative means of vaccination. Nasal associated lymphoid tissue (NALT) is responsible for stimulation of the immune response. NALT is anatomically similar to GALT but, has more well developed lymphoid follicles than GALT and also consists of numerous M cells, dendritic cells and a few goblet cells. M cells of NALT function similar to those found in peyers patch. In nasal vaccination, the antigen produces either an immune response or a tolerance (Sminia, et al., 1999 & McGhee et al., 1992). Partidos reported that this decision is governed by physical nature of the antigen and its interaction with NALT (Partidos, et al., 2000). Although tolerance is unwanted in case of
vaccination, it can be a very effective tool against auto-immune diseases (Czerkinsky, C. et al., 1999). It was observed that particulate antigens show a mucosal immune response while soluble antigens produce a systemic immune response. This difference is due to the easy of diffusion of soluble antigen through the nasal epithelium that presents it to the superficial cervical lymph nodes and stimulates IgG production, while M cell in NALT selectively take up particulate antigen and stimulate IgA antibody production (Van der Lubben, et al., 2001, Kuper, 1992).

Advantages of nasal vaccination over oral vaccination:

i) Higher permeability of mucosal membrane as compared to gastro-intestinal mucosal membrane (Ugwoke, et al., 2001).

ii) Protection of the antigen from exposure to low pH.

iii) Low enzymatic activity as compared to GIT (Csaba, et al., 2009).

iv) NALT consists of dendritic cell which are said to be the most potent antigen presenting cells (Jahnsen, et al., 2004).

v) Nasal vaccination results in stimulation of antigen specific Th1 and Th2 cell mediated IgA immune response (Yanagita, M et al., 1999).

vi) Nasal route for vaccination produces faster and stronger immunity as compared to its oral counterpart (Wu, H. Y et al., 1997).

vii) NALT retains long term memory hence subsequent exposure of antigen results in a quick immune response.

viii) Intranasal vaccination can also stimulate cytotoxic T lymphocyte response which plays an important role in killing of intracellular pathogen like, virus and reckettia (Moore, A et al., 1995).

2.8.3 Sublingual Route: This route of administration is commonly used for allergen vaccination (Olaguibel, J. M et al., 2005). Now days sublingual immune-therapy (SLIT) has become popular for treatment of type I allergies (Van Overtvelt, L et al., 2006). This is based on desensitization of patient after administration of allergen by sublingual route. It is safer and more effective than subcutaneous route.

2.8.4 Pulmonary Route: In this route of administration, the vaccine is administered through an aerosol system to the alveoli. Bronchus associated lymphoid tissue (BALT) is responsible
Characterization of HPV candidate antigen and Development of novel vaccine delivery system

for stimulation of this immune response. Immunization using this route has been reported by Amoriji et al. (Amorij, et al., 2007).

2.8.5 Rectal Route: Rectal route of administration can be used as an alternative to the oral route, because it not only avoids the exposure of antigen to low pH and proteolytic enzymes but is also effective in inducing specific immune response in intestine and distal sites of CMIS. It has some serious limitation like expulsion of vaccine after administration and lack of patient compliance.

2.8.6 Vaginal Route: This is an attractive route for protection of female reproductive track against sexually transmitted infections such as HIV, human papillomavirus (HPV) and Herpes simplex virus (HSV). This is useful for stimulation of local immune response but is not a preferred form. This is because immune response is under the hormonal control and hence changes depending upon the stage of the menstrual cycle. It has been proved that intranasal immunization stimulates the immune response in vagina more effectively than vaginal vaccination (Di Tommaso, et al., 1996).

2.8.7 Ocular Route: Ocular route can be used for effective vaccination against a number of infectious pathogens affecting the ocular tissue. As external part of the eye shares common mucosal characteristic, it can be employed as an alternative to other mucosal routes of vaccination. Recent findings of the ocular mucosal immune system (OMIS) may lead to the effective design of vaccines against ocular pathogen (Nesburn, et al., 2006). Conjunctiva associated lymphoid tissue (CALT) is involved in its immune response and responsible for local as well as systemic protection against pathogen. Unlike oral, there is no exposure of antigen to harsh environment like low pH. Ocular vaccination does not redirect antigen into central nervous system like nasal route (Seo, et al., 2010).

2.9 Nanocarrier Based Approaches for Transmucosal Vaccine Delivery: Nanocarriers are drug carrier systems which range, usually from 1 to 1000 nm. Most of these nanocarriers are named by their systems, e.g, nanoemulsions, nanotubes, nanosuspensions and nanoparticles. Nanocarriers are of great scientific interest because of their small size. It is well accepted fact that properties of a material changes as its size approaches the nanoscale, hence nanoparticles exhibit a number of special properties relative to the bulk material. Since nanocarriers have a very high surface area to volume ratio, diffusion rate of nanocarriers is tremendous. Many
Characterization of HPV candidate antigen and Development of novel vaccine delivery system

nanocarriers have been used for drugs as well as vaccine delivery (Singh, et al., 2010 & Farokhzad, et al., 2009).

At present, nanocarrier based vaccines enjoy the following advantages over traditional vaccine carriers:

i) Vaccines delivered in nanocarrier forms are more efficiently localized to the targeted tissues.

ii) Nanocarrier based vaccines are more efficiently taken up by the antigen presenting cells such as macrophages, dendritic cells because of their comparable dimensions to the pathogen. Hence, delivery of antigen to the antigen presenting cells is facilitated (Shahiwala, et al., 2007).

iii) It has been reported that, nanocarrier based vaccines produce greater immune response as compared to conventional vaccines (Griffiths, et al., 1997).

iv) With some new techniques it is possible to produce stronger protective immunity with single dose using nanocarriers such as ImuXen Tech.

v) Bioavailability of an oral vaccine intended for systemic immune response can be increased using nanocarrier based systems (Arbos, P et al., 2004).

vi) In addition to efficient delivery, nanocarriers also act as adjuvant and provoke the immune response.

Figure 4: Schematic representation of uptake of antigen by follicle associated epithelium. (Jain et al., Current Nanoscience, 2011, Vol. 7, No. 2)
Nanocarriers containing antigens are either taken up by M (microfold) cells that are present between the mucosal epithelial or intraepithelial dendritic cells (DCs). These M cells process the antigen and forward it to the antigen presenting cells (APCs) such as macrophages and antigen carrying dendritic cells (Fig. 4). These cells present the antigen not only to the local B or T cell, but also leave the peyers patches and reach the systemic circulation via the thoracic duct and present antigen to distant B or T cell. Thus immune response is observed locally as well as at distant sites. Alternatively, antigens are directly taken by intra-epithelial DCs and forwarded to the antigen carrying DCs. A recent review by Borges et al., provides a mechanistic aspect of the events within mucosal tissues which lead to protective mucosal immune responses. In addition to the M cells, inductive mucosal site of gastrointestinal tract also contains the mucine producing glandular cells which protect the gastrointestinal mucosa by secreting thick mucus layer over it. However, this act as a barrier by entrapping nanocarriers, causing agglomeration and hence limits the uptake of antigen by M cell.

### 2.9.1 Classification of Nanocarriers:

On the basis of physical nature, nanocarriers are classified as particulate nanocarriers, vesicular nanocarriers, and other miscellaneous types.

(Jain et al., Current Nanoscience, 2011, Vol. 7, No. 2)
2.9.2 Factors Affecting The Performance Of Nanocarriers: There are various factors which directly or indirectly affect the performance of nanocarriers i.e. their ability to deliver the antigen to the antigen presenting cells. Many of these factors affect the release rate of antigen and/or uptake of nanocarriers by M cells or APCs. These factors include the particle size, hydrophobicity/ hydrophilicity, stability in biological environment, glass transition temperature, crystallinity of polymers, and ratio of co-polymers, bioadhesiveness and the nature of additives. These factors have been discussed below. 

Particle Size: It has been observed that, absorption depends on the surface area of polymer and as particle size decreases, surface area increases. Hence, being in nanosize, nanoparticles are absorbed rapidly (Jani, et al., 1992). It was concluded that decrease in particle size below the 1 micrometer, results in uptake of particles by APCs which result in an increase in the immune response (Florence, et al., 2005). The intrinsic capacity of nanoparticles to interact with mucosal surface also depends upon the particle size (Florence, et al., 2001), smaller the size, higher is the interaction with mucosal cells (Jani, et al., 1990). Uchida et al., demonstrated the effect of particle size on immunity with help of PLG microparticles entrapping Ovalbumin. They reported that the particles up to 4 micro meter induced systemic immunity while particles greater than 7 micrometer induced mucosal immunity (Uchida, et al., 1994).

a. Hydrophobicity/Hydrophilicity: It has been demonstrated that uptake of hydrophobic nanocarriers by mucosal epithelia is higher as compared to hydrophilic nanocarriers. In 1988, Kreuter et al. showed that increasing hydrophobicity of nanocarrier results in increased adjuvant activity (Kreuter, et al., 1988). Hydrophobic nanocarriers include nanotubes, solid lipid nanoparticles and widely used polymeric nanoparticles, namely, PLG and PLGA that also enjoy the advantages of established safety. Hydrophobic nanocarriers suffer from insolubility in biological fluids. Since most antigens (protein/polypeptides, polysaccharides) are hydrophilic in nature, loading them into hydrophobic polymer is difficult. Hydrophilic nanocarriers overcome the limitation of hydrophobic nanocarriers. Being hydrophilic, they are soluble in biological fluids and loading of antigen is easy. They have affinity towards mucosal surface which is an important feature as far as transmucosal delivery is concerned (Calvo, et al., 1997, Jose Alonso et al., 1999 & Csaba, et al., 2006), but
they deliver antigens within a short time. This class includes chitosan nanoparticles, dendrimers, hydrogel nanoparticles and polymeric micelles. The study carried out with Diphtheria toxoid by Jani et al. suggests that highly hydrophilic or highly hydrophobic suspension failed to produce any adjutancy. Intermediate hydrophobicity produced the highest immunological response. The same group of workers also reported the alteration in the antibody isotype profile by changing the hydrophile-lipophile balance (HLB) of formulations (Jani, et al., 1990).

**b. Stability in Biological Environment:** Unless particles are stable in biological fluids, they are not able to deliver the antigen to targeted cell. In this sense hydrophilic nanocarriers are better suited for mucosal vaccine delivery, since they are stable in biological fluids which is aqueous in nature (Csaba et al., 2006).

**c. Glass Transition Temperature and Crystallinity of Polymers:** Glass transition temperature (Tg) is the temperature at which polymer changes from a glassy state to a rubbery state. Glass transition affects release properties of nanoparticles. Transition from glassy state to rubbery state results in an increase in the free volume of amorphous phase which increases the antigen release from nanocarriers. This is because; solubility of antigen in the polymer is inversely proportional to the amorphous content in the polymer. An increase in the amorphous content leads to an increase in release rate (Norris, et al., 1998).

**d. Ratio of Co-Polymer:** Changing the ratio of co-polymer, causes a change in the hydrophilic/ hydrophobic nature of nanocarriers which affects the release rate of the antigen, particle size, antigen loading and entrapment efficiency of nanocarriers. For example, lactide: glycolide ratio in the PLGA polymer has a significant effect on degradation kinetics of nanoparticles and hence antigen release profile (Rajapaksa, et al., 2010).

**e. Bioadhesiveness:** This property is essential for the delivery of vaccines using polymers. It in fact depends on hydrophilicity of polymer. Good bioadhesiveness of polymer results in better interaction of nanocarriers with mucosal membrane (Takeuchi, et al., 2001 & Prego, et al., 2005).

**f. Nature of Additives:** Additives involved in fabrication of nanocarriers affect their performance. Sugars when incorporated into hydrophobic nanocarrier, increase antigen release rates. This can be explained by the following example. In the case, PLGA based
hepatitis B surface antigen (HBsAg) vaccine nanoparticles, addition of trehalose increases
the release rate of HBsAg as compared to nanocarriers without trehalose (Gupta, et al., 2006 & Jaganathan, et al., 2004). This is because trehalose; being a sugar; dissolves rapidly from
the hydrophobic PLGA matrix, leaving a porous matrix, which releases the antigen at a faster
rate than nonporous matrix (PLGA without trehalose). Some of the additives affect the
particle size, Zeta potential, surface hydrophobicity, polydispersity index and protein loading.
This can be best explained by the example of PVA. Sahoo et al., have studied effect of
residual PVA on physical properties of PLGA nanoparticles and its cellular uptakes (Sahoo,
et al., 2002). It was found that PVA affected above mentioned pharmaceutical properties of
PLGA particles and hence their performance.

2.9.3. Essential Characteristics of Nanocarriers for Transmucosal Vaccine Delivery:
There are three major characteristics to be fulfilled by nanocarriers when used for vaccine
delivery. These are as follows -

a. Protection of Antigen: Since most antigens are proteins/polypeptides, polysaccharides
and/or DNA which are delicate macromolecules, can be degraded in biological system such
as GIT upon exposure to harsh pH condition and enzymatic activity. Hence protection of
antigen is prime requirement for nanocarriers (Csaba, et al., 2006).

b. Transport across Mucosal Cell: Proteins/polypeptides or polysaccharides are poorly
transported across mucosal epithelial. Hence nanocarriers should facilitate their transport
across mucosal surface (Csaba, et al., 2006).

c. Delivery of Antigen to APCs: Nanocarriers should deliver antigens to immune-competent
cells such as macrophages and dendritic cells so that they cells are able to recognize antigen
and activate the immune system (Stuart, et al., 2005).

2.9.4. Various Nanosystems for Transmucosal Vaccine Delivery: Several nanocarriers
have been reported in the literature for the delivery of vaccines. A review of these is
presented in this section.

a. Chitosan-Based Nanocarriers: Many studies have demonstrated that it is very easy to
obtain nanoparticles from chitosan (CS) and these are very efficient as well as nontoxic
absorption enhancer for mucosal administration of vaccines, proteins and peptides (Calvo, et
are well suited nanocarriers for transmucosal vaccine delivery due to their mucoadhesive properties, attributed to polycationic nature of the polymer, which result in ionic interaction with negatively charge mucosal surface (Ilium, et al., 1998, Janes, et al., 2001, Thanou, et al., 2001, van der Lubben, et al., 2001, & Hejazi, et al., 2003). In 2002, Vila et al., reported the very first application of CS nanoparticles for transmucosal vaccine delivery. They found that CS based tetanus toxoid (TT) nanoparticle vaccine when given by intranasal route showed a high level of systemic as well as mucosal immune response as compare to conventional TT vaccine (Vila, et al., 2002). Calvo et al., modified the CS nanoparticles by PEGylation and found that CS-PEG nanoparticle based diphtheria vaccine showed a better response than non-PEGylated CS. Other modified CS nanoparticles based vaccines also showed good immune response and proved to be a better alternative.

However, in case of oral delivery of chitosan based formulation, release pattern of antigen was found to be erratic and was unreliable because of high solubility of chitosan in acidic medium (Hejazi, et al., 2002). To overcome this problem, Jain et al., encapsulated the chitosan nanoparticles in lipid vesicular system such as liposomes and niosomes. They reported high IgA as well as IgG titers, using bovine serum albumin (BSA) loaded chitosan nanoparticles encapsulated in liposomes and niosomes in albino rats (Jain, et al., 2006). In 2006, Borges et al., carried out a study using alginate coated chitosan nanoparticles based ovalbumin vaccine on rats, and reported enhanced uptake and high IgA antibody titers (Borges, et al., 2006). Chitosan has strong adjuvant effect comparable to Freund’s adjuvant and this was demonstrated with zinc-chitosan particles and an emulsion containing chitosan (Seferian, et al., 2000).

b. PLGA and PLA Based Nanocarriers: These polymers have attracted researchers because of their biodegradability, sustained release property and hence its potential use for single dose vaccine formulations (Alonso, et al., 1994, Thomasin, et al., 1996). Poly (lactide-coglycolide) nanoparticles are the most studied nanocarriers for oral delivery and many reports have been published which discuss their preferable uptake by peyers patches (Gupta, et al., 2007). But the limitation of poor stability in biological fluid, results in instability in encapsulated vaccines as well. This limitation can be overcome by modifying or blending these hydrophobic particles with hydrophilic groups or polymers such as PEG, poloxamers
and poly-vinyl alcohol (PVA). Encapsulation of antigen into polymer leads to the alteration in integrity and immunogenicity of antigen due to the exposure to organic solvent, high shearing stress and low pH environment causing degradation of polymers. This can be overcome by adsorbing the antigen on polymeric system rather than encapsulation. Polymeric lamellar substrate particles (PLSP) are an outcome of such stabilization (Jabbal-Gill, et al., 2001).

c. Solid Lipid Nanoparticles: These are hydrophobic nanocarriers which are made up of solid lipid core coated with monolayer of phospholipids and dispersed in an aqueous system. Solid lipid nanoparticles (SLN) have similar structure that of nano-emulsions which consist of liquid lipid core instead of solid lipid core in SLN which consist of solid lipid or mixture of lipids. They have an advantage over nano-emulsions that their degradation rate in the body is very slow which can be further reduced by using sterically stabilizing surfactants (Olbrich, et al., 1999). Thus SLN can be used for sustain release of antigen which results in a long lasting immune response. Possible use of SLN as nanocarriers for vaccine delivery has been reported in literature (Almeida, et al., 1997). In 2007, Rao and Koteswara invented polymeric SLN for mucosal delivery of antigen (Rao, et al., 2007).

Advantages of SLN over other nanocarriers includes

I. Toxicity is negligible as compared to polymeric nanoparticles (Muller, et al., 1996).

II. Suitable for controlled release of antigen hence can be used for single dose formulations of vaccine.

III. SLN can be used as effective non-viral transfection agent (Olbrich, et al., 2001, Pedersen, et al., 2006).

d. Liposomes: Liposomes are widely used to encapsulate protein and DNA based vaccines. Liposomes as carriers of antigens and adjuvants have been well reviewed (Alving, et al., 1994). It has been reported that liposomes show immunomodulatory effect when administered as vaccine adjuvant (Chikh, et al., 2002, Sprott, et al., 2004, Alving, et al., 1992, Gregoriadis, et al., 1994, Ambrosch, et al., 1997). Enhanced immune response of liposomally encapsulated ricin toxoid vaccine for intra-tracheal administration was reported. Immunization studies with liposomal tetanus toxoid vaccine showed that nasal route is best suited for mucosal vaccination (Tafaghodi, et al., 2006).
Zhou and Neutra, delivered antigen to mucosa associated lymphoid tissues by oral route using liposomes as nanocarriers (Zhou, et al., 2002). They also tried ferritine antigen by rectal route and concluded that rectal administration also gives effective mucosal immunity (Zhou, et al., 1995). Another study carried out using gangliosides GMI encapsulated liposomal vaccine in mice, reported higher IgA antibody titer (Watarai, et al., 1998).

Liposomes enjoy the advantages of being biocompatible, biodegradable, easy surface modification but suffer from instability in GIT that leads to leakage of encapsulated antigen and its degradation. This can be overcome by development of polymerized liposomes (Chen, et al., 1996). Vaccines based on polymerized liposomes for oral administration have been patented (Okada, et al., 1998). One more modification suggested by Lasic et al., was stealth liposomes in which surface of liposomes were coated with polyethylene glycol. This modification results in an increased half-life of liposomal vaccine (Lasic, et al., 1999). Despite these advances, liposomes suffer from disadvantages of being expensive, ingredient instability (chemical instability of phospholipids) due to their predisposition to oxidative degradation, special storage and handling conditions and variable purity of natural phospholipids.

e. Bilosomes: In 2004, Mann et al., developed non-ionic surfactant vesicle (NISV), having liposome- like structures and stabilized them with bile salts for the oral delivery of vaccines. These were called bilosome. Bilosomes differ from the liposomes and niosomes in term of their composition, chemical stability and storage conditions. In order to avoid the problems during GI transit, bilosomes were developed which not only prevented antigens from degradation, but also enhanced mucosal penetration. Bilosome based vaccine produced both systemic as well as mucosal immune response which was equivalent to immune response produced by subcutaneous route (Mann, et al., 2004). Two years later, new bilosome based vaccine formulation i.e. tetanus toxoid using vesicles containing bile salts for oral delivery were studied (Mann, et al., 2006). Nanotoxicity and adjuvant effect of NISV was established (senior, et al., 2001). The ability of orally administered bilosome based vaccines to induce systemic immune responses in mice, using a standard antigen (bovine serum albumin), a synthetic measles peptide and an influenza sub-unit vaccine were studied. Immune response of above vaccine formulations was increased by incorporating bile salts (in particular
Characterization of HPV candidate antigen and Development of novel vaccine delivery system

deoxycholate). Bilosome based measles peptide vaccine stimulated a specific cell mediated response. Orally administered bilosome based influenza sub-unit vaccine induced systemic immune response same as that produced by parenterally administered vaccine containing the same quantity of antigen (Conacher, et al., 2001).

2.10. Niosomes: Niosomes are non-ionic surfactant vesicles obtained on hydration of synthetic non-ionic surfactant, with or without incorporation of cholesterol or other lipids. They are formed from the self-assembly of non-ionic amphiphiles by hydration in aqueous media resulting in closed bilayer structure (Figure 5).

![Figure 5: Niosome structure](image)

Niosomes are non-ionic surfactant vesicles (NISV) formed from the self-assembly of synthetic nonionic surfactants, with or without the incorporation of cholesterol or other lipids in aqueous media which result into closed bilayer vesicular structures. Synthetic non-ionic surfactants such as Span 60 are amphiphilic in nature, and form vesicular systems similar to liposomes. Hence niosomes are classified as amphiphilic nanocarriers. Niosomes overcome all the disadvantages of liposome discussed previously. Niosomes enhance immune response against bovine serum albumin (BSA) in Balb/c mouse and this was comparable with Freund’s complete adjuvant (FCA) (Brewer, et al., 1992). Niosomes have been reported to act as a vaccine adjuvant and promote the Th1 immune responses when administered intraperitoneally to PBL-SCID mice (Walker, et al., 1996). Rentel et al. have formulated various lyophilized niosome preparations consisting of sucrose esters, cholesterol and diacetyl phosphate. They encapsulated ovalbumin in niosomes of two different compositions which were given perorally to Balb/c mice. They compared the immune response of this formulation and concluded that administration of ovalbumin and empty niosomes alone did not stimulate similar immune response as that of ovalbumin encapsulated niosome. Among
the various compositions of ovalbumin encapsulated niosomes, 70% stearate sucrose ester, 30% palmitate sucrose ester (40% mono-, 60% di:tri-ester) niosomes showed significant immune response (Rentel, et al., 2005).

Jain et al. have developed mannosylated niosomes as nanocarriers for oral delivery of DNA vaccine for the induction of humoral, cellular as well as mucosal immunity against Hepatitis B in Balb/c mice. They modified niosomes by polysaccharide o-palmitoylmannnan (OPM). The rationale behind this was to protect the niosomes from bile salt which was responsible for dissolution and also from enzymatic degradation in the gastrointestinal tract. It also enhanced the affinity of niosomes towards the M cell of Peyer’s patches. In this study, they reported that, systemic immune responses elicited by orally administered OPM coated niosomal formulations were lesser than that elicited by intramuscularly administered naked DNA and pure HBsAg. But level of immune response achieved was far above the clinically protective limit for humans (i.e. 10mIU/ml). OPM coated niosomes have advantages over intramuscularly administered naked DNA and recombinant HBsAg that they produced humoral (both systemic and mucosal) as well as cellular immune response upon oral administration. Intramuscularly administered naked DNA and recombinant HBsAg did not induce mucosal immunity while cellular response (cytokines level) was not shown by pure HBsAg treated animals (Jain, S et al., 2005). Mannosylated niosome based tetanus toxoid vaccine for oral delivery was also developed and immune response was compared with orally administered alum-adsorbed TT and orally administered plain uncoated Niosomes based TT. It was observed that OPM coated Niosome based vaccine formulation produced greater systemic as well as mucosal immune response as compared to plain uncoated niosomes as well as alum-adsorbed TT (Jain, et al., 2006).

2.10.1. Chemical composition of Niosomes:

a. Surfactants: Following the application of some forms of energy such as mechanical or heating, the formation of niosomes is a self-assembly process due to high interfacial tension between aqueous medium and the lipophilic alkyl chain(s) resulted in the association of non-ionic surfactant monomers into vesicles. Concurrently, the hydrophilic head groups of amphiphilic molecules make water mediated interactions counter the previous formed force eventually results in bilayer formation. Alkyl ethers, alkyl esters, alkyl amides, fatty acids
and amino acids are the main non-ionic surfactant classes used for niosome production. However, the most frequently used surfactants in niosomes formulations are sorbitan monoesters (Spans®, Fig. 6). The versatility of compounds capable of forming vesicle is due to the presence of different and various polar head groups attached to saturated or unsaturated alkyl chain(s) composed of 12 to 18 carbon atoms (C12-C18).

![Chemical structure of frequently used surfactants in niosomes formulations, sorbitan monoesters (Spans®).](Pardakhty A, et al. 2013, Nanomed. J., Vol. 1, No. 1)

Formation of niosomes requires an amphiphilic molecule composed of two main parts, a polar or hydrophilic head group and a non-polar or hydrophobic tail. This is obviously the ordinary structure of surfactant molecules, but in many cases the presence a wedge-shaped molecule such as CHOL is essential for turning the micellar structure of surfactant aggregates to bilayer arrangement. The lipophilic moiety of amphiphile molecule may contain one (Varshosaz et al., 2003), two (Okahata et al., 1981) or three (Jain et al., 1995,
Chapter 2: Review of Literature

Jain et al., 2005, Yoshioka et al., 1994) alkyl or perfluoroalkyl (Zarif et al., 1994) groups or in some cases, a single steroidal group.

b. Methods of niosome preparation: Generally there are two strategies for niosome or liposome preparation; the first set involves dissolving the whole lipids in organic solvent(s) for molecular level mixing of the bilayer constituents, then removing the organic solvent and hydration of formed lipid thin films or surfaces by an aqueous medium. Film hydration (Pardakhty et al., 2012), reverse phase evaporation (REV) (Balasubramaniam et al., 2002), ether injection (Devaraj et al., 2002, Rogerson et al., 1987), dehydration rehydration (DRV) and solvent evaporation from double emulsion droplets are the most common methods in which an organic solvent is exploited. The second strategy involves the direct mixing of lipids and hydration medium, usually in high elevated temperature, which has the advantage of not having the hazardous effects of residual of organic solvents on entrapped substance or biologically applied environments. The widely used and well-documented methods for vesicle production include heating and sonication of lipid (Suwakul et al., 2006), homogenization of lipids (Zidan et al., 2011), lamellar liquid crystal transformation (Liu, et al., 2007), heating (Mozafari method), supercritical CO2 (Manosroi et al., 2008), inert gas bubble (Talsma et al., 1997), microfluidic hydrodynamic focusing (Lo et al., 2010) and the electroformation of vesicles which utilizes alternating electric fields to generate vesicles in aqueous solutions of the amphiphilic molecules (Okumura et al., 2011).

2.10.2 Factors Influencing the Immunological Properties of Niosomes

i) Surfactant Alkyl Length: Studies with a variety of surfactant based adjuvants have indicated that adjuvanticity may be affected by the fatty acid composition of the surfactant hydrophobe (Gall, 1966, Bomford, 1981). Analysis of OVA specific IgG1 titers in plasma samples collected 2 weeks after secondary inoculations with OVA clearly demonstrates that NISV prepared from all alkyl chain length surfactants have adjuvant activity compared with OVA (p<0.01). Of these, only mice inoculated with OVA prepared in C14:0 (p<0.025) or C18:0 (p<0.025) had levels of antibody significantly lower than C16:0 monopalmitoyl glycerol. Analysis of the Th1 associated IgG2a subclass of antibody at the same time point revealed that, in contrast to IgG1 levels, NISV prepared from C18:0 monostearoylglycerol failed to produce an adjuvant effect. This level of antibody production was also significantly
less than that induced by NISV prepared from monopalmitoyl glycerol (p<0.025). It has also been previously demonstrated (Brewer and Alexander, 1992) that merely mixing antigen with empty NISV is not sufficient for adjuvant activity, therefore vesicles which remained more stable in vivo and retained more of their encapsulated antigen would be more effective adjuvants, possibly due to greater capacity to target antigen to antigen presenting cells or ability to form short lived depots. Therefore, the alkyl chain length dependent differences could be explained in terms of heterogeneity of bilayer permeability for antigen or stability of different formulations in vivo. However, studies with non-vesicular formulations have also identified that the number of carbon units in the hydrophilic chain of a surfactant has important effects on adjuvanticity.

ii) **Surfactant linkage**: Another feature of surfactants which may affect adjuvanticity is the nature of the linkage between the hydrophobe and the hydrophile. Work by Hofland and colleagues (Hofland and Boustra, 1991) indicate that NISV prepared from ether linked surfactants have tenfold higher toxicity than vesicles prepared from an analogous ester linked surfactant.

iii) **Vesicle surface charge**: The role of surface charge in the adjuvant effect of liposomes has been variously reported as unimportant (Agrewala & Owais, 1996) or in other cases significant (Allison and Gregoriadis 1974; Kraaijeveld and Schilham, 1984; Aramaki and Fujii 1994). When preparing vesicles from non-ionic surfactants it is important to include a charge producing amphiphile which provides charge repulsion between vesicles thereby preventing lipid aggregation and vesicle disruption. However the role that the charged amphiphile, in this case DCP, has in the adjuvant effect is unclear.

iv) **Stability of NISV**: In addition to the limited range of immune responses induced by Alum, the application of this adjuvant, particularly to third world vaccines, is hampered by the requirement for 'cold chain' storage. Furthermore, Alum adsorbed vaccines are not compatible with lyophilisation or storage at -20°C which would be desirable for the long term storage of subunit vaccines. Adjuvant activity was assessed by immunizing mice with the various preparations, administering booster inoculations of soluble protein two weeks later, followed by assay of plasma antibody production. From the data it is clear that NISV could be stored for up to 10 months in all of the conditions tested without appreciable loss of
Characterization of HPV candidate antigen and Development of novel vaccine delivery system

adjuvant activity compared with fresh NISV preparations. However, following storage for 18 months at room temperature, variations in the adjuvant activity between the NISV preparations maintained under the various storage conditions became evident. Of the conditions tested, storage at room temperature resulted in the greatest loss of adjuvant activity while storage of the lyophilized NISV containing sucrose at room temperature resulted in the least (Figure 4b). The incorporation of sucrose appeared to have an important effect as the preparation lyophilized in normal buffered saline had in comparison appreciably lower maintenance of adjuvant activity. This could be attributed to the action of sucrose as a cryopreservant (Crowe and Crowe, 1984; Strauss and Hauser, 1986). It is very clear from many studies that NISV are very robust adjuvants and can retain adjuvant activity following storage for up to 10 months regardless of the conditions of storage, even at room temperature.

v) Toxicological properties of NISV: The toxicity of adjuvant formulations is a critical factor given that vaccines are a prophylactic measure generally administered to a healthy population. However, it has been suggested that stimulation of the immune response by adjuvants is pharmacologically a toxic reaction and therefore adjuvant selection represents a choice between toxicity and effective adjuvant activity (Gupta and Relyveld, 1993).

Analysis of the site of injection in rats 28 days after intramuscular inoculation of NISV (30 or 90 mg /Kg), revealed a slight lymphocytic infiltrate at the site of injection with no local persistence of NISV. This is consistent with studies in mice where following subcutaneous administration in the footpad, NISV could be found in the draining lymph node after 30 minutes and the majority of NISV cleared from the site of injection 24 hours later. In contrast, when rats were administered 8 mg/Kg Alum, substantial lymphocyte infiltration and chronic granuloma formation occurred at the site of injection. In dose escalation studies, only doses of NISV in excess of 575 mg/Kg persisted at the site of injection for longer than 14 day.

Although frequently proposed as a mechanism of action for adjuvants, the importance of the antigen depot and local inflammation induced at the injection site in mediating adjuvant activity has been the subject of conjecture in the literature for a considerable time. In fact, it
has even been considered that production of a local granulomatous response, even when using Aluminium hydroxide in human vaccines, is a necessary requirement for effective adjuvant activity to be induced (WHO 1976). However, it has also been well demonstrated that excision of the antigen depot does not affect the overall specific immune response (Lascelles and Eagleson, 1989).

In contrast, excision of both the draining lymph node as well as the site of injection does result in a dramatic decrease in antibody responses (Lascelles and Eagleson, 1989). These studies indicate that the ability of adjuvants to stimulate cells in the draining lymph node is more important in sustaining antibody production than the reactions occurring at the site of injection.

2.10.3 Niosomes in drug delivery: Nano-niosomes are currently used as versatile drug delivery systems with many pharmaceutical applications, including for oral, pulmonary, transdermal, parenteral, vaginal, nasal and ophthalmic route of administration.

a. Oral route: The in vivo distribution study of *Ginkgo biloba* extract nano-vesicles composed of Tween 80/Span 80/CHOL showed that the flavonoid glycoside content in heart, lung, kidney, brain, and blood of rats treated with niosomal carrier system was greater than those treated with the oral *Ginkgo biloba* extract tablet (Jin et al., 2013). Mean particle size of mentioned niosomes was in nano size range (141 nm) which resulted in both altered pharmacokinetic behavior and in vivo distribution of the plant extract. Di Marzio et al., (2012) prepared polysorbate 20 nano-niosomes for oral delivery of unstable or poorly soluble drugs by film hydration associated with sonication in order to reduce the size down to sub-micron range. These vesicles were stable in different pH and in simulated gastrointestinal media with high mucoadhesion properties.

b. Parenteral route: Anticancer chemotherapy by using vesicular system have many benefits such as reduced organ toxicity (O’Brien et al., 2004), enhanced antineoplastic efficacy, prolonged circulation of vesicular carriers (Cosco et al., 2009) and less mortality in patients. On the basis of these pharmaceutical and clinical facts, innovative niosomes made up of α,ω-hexadecyl-bis-(1-aza-18-crown-6) (bola), Span 80 and cholesterol (2:5:2 molar ratio) were prepared as suitable delivery systems for the administration of 5-fluorouracil (5-FU) (Cosco et al., 2009).
Magnetic drug targeting to a specific organ or tissue is proposed on the assumption that magnetic fields are harmless to biological systems. On the basis of this hypothesis, Tavano et al. (Tavano et al., 2013) prepared Tween 60 and Pluronic L64 doxorubicin loaded magnetoniosomes with low toxicity and high targeting potential. Reducing the mean volume diameter and PEGylation of hydroxyl-camptothecinniosomes resulted in stealth effect and high antitumor activity of this chemotherapeutic agent (Shi et al., 2006).

Ribavirin niosomes were prepared by thin film hydration method using Span 60, CHOL, and DCP for liver targeting purpose (Hashim et al., 2010). The results showed that the niosomal formulation significantly increased ribavirin liver concentration (6 fold) in comparison with ribavirin-free solution. Mukherjee et al. (2007) showed the superior stability and encapsulation efficiency of acyclovir in 200 nm niosomes in comparison to soya L-α-lecithin liposomes. They concluded that niosome could be a better choice for intravenous delivery of acyclovir.

c. Vaginal route: Two kinds of entrapped insulin vesicles with Span 40 and Span 60 were prepared by lipid phase evaporation and sonication methods with particle sizes of 242.5 nm and 259.7 nm, respectively (Ning et al., 2005). They concluded that vaginally administrated nano-niosomes might be a good carrier for protein drugs such as insulin.

2.10.4 Niosomes in vaccine delivery:

a. Protein subunit vaccines: Development of new safe and effective vaccines is an important goal for many research groups in all over the world. Subunit proteins or DNA of various organisms are safer than live organism-based vaccines even they may show less efficacy. The use of adjuvanted systems have proven to enhance the immunogenicity of these subunit vaccines through protection (i.e. preventing degradation of the antigen in vivo) and enhanced targeting of these antigens to professional antigen-presenting cells (Obrenovic et al., 1998).

i) Brewer and Alexander (Brewer et al., 1992) reported the first application on niosome antigen delivery for immunization of Balb/c mice against bovine serum albumin (BSA). They deduced that niosomes were potentially better stimulators of the Th1 lymphocyte subset than was Freund's complete adjuvant and by inference, potent stimulators of cellular immunity.
ii) Hassan et al. (Hassan et al., 1996) showed better immunogenicity with herpes simplex virus 1 antigen encapsulated in 1-mono palmitoyl glycerol (MP)/CHOL/DCP niosomes in mice. On the other hand, partial protection against homologous (type 2 herpes simplex virus HSV-2) challenge infection afforded to mice by HSV-2 antigen encapsulated niosomes (Mohamedi et al., 2000) shows the importance of composition and method of niosomal adjuvant formulations.

iii) Yoshioka et al. (Yoshioka et al., 1995) formulated Span/CHOL/DCP niosomes containing tetanus toxoid (TT) emulsified in an external oil phase to form a vesicle-in-water-in-oil (v/w/o) formulation. Initial studies of the system in vivo using cottonseed oil as the external oil phase, showed enhanced immunological activity Encapsulation of BSA or haemagglutinin (HA) in v/w/o emulsion was also reported by Murdan (Murdan et al., 1999).

iv) Immunogenicity studies showed that the v/w/o gel as well as the water-in-oil (w/o) gel as control, possess immunoadjuvant properties and enhance the primary and secondary antibody titres (of total IgG, IgG1, IgG2a and IgG2b) to HA antigen. Chambers et al. (2004) reported a single subcutaneous dose of killed *Mycobacterium bovis* BCG in Brij® 52-based nanoniosomes (Novasome TM) protected guinea pigs from lethal tuberculosis.

v) Vangala et al. (Vangala et al., 2006) incorporated three different protein antigens in positively charged niosomes made from MP/CHOL/α,α′-trehalose 6,6′-dibehenate (TDB) or MP/CHOL/TDB/dimethyl-dioctadecylammonium (DDA). Antigens encapsulation led to increase in size of vesicles from submicron to larger (1-2.7 μm) ones which may be due to the high molecular weight of antigens, in addition to their high hydrophobic nature, causing the association of the proteins with the hydrophobic regions of the vesicle bilayers and possibly encouraging a degree of vesicle fusion or influencing the packing arrangements of the surfactants. Their results suggest that both DDA- and MP-based vesicular systems may be useful in enhancing the immunogenicity of the subunit vaccines, especially with the subunit antigen Ag85B-ESAT-6 against tuberculosis, for which a high cell-mediated Th1 immune response is essential (Vangala et al., 2006).

vi) Vangala et al., (2006) also reported DDA formulations incorporating TDB which showed markedly increased Hepatitis B surface antigen specific splenocyte proliferation and elicited
cytokine production concomitant with a strong T cell driven response, delineating formulations that may be useful for further evaluation of their clinical potential.

vii) Ferro and Stimson (Ferro et al., 1998) used a gonadotrophin releasing hormone (GnRH) analogue, GnRH-glycs, linked to different carrier molecule and encapsulated in NSV formulations to immune-neutralisation of GnRH in male Sprague-Dawley rats. The results were encouraging to use NSVs as a nontoxic immune adjuvant. Then, a modified GnRH peptide (CHWSYGLRPG-NH2) was conjugated to TT and formulated with different adjuvants such as C18EO2/CHOL-/DCP niosomes (Ferro et al., 2004). The best castration effect, depicted in production of IgG2b antibody, was not as well by nano-niosomes as compared to sustained release poly(lactide-co-glycolide)/triacetin (PLGA) formulation.

viii) A promising immunization effect was reported by Lezama-Davila (LezamaDávila CM et al., 1999) in C57BL/10 mice immunized with L. m. mexicana leishmanolysin (gp63).

ix) For developing non parenteral niosomal vaccines, Rentel et al., (Rentel et al., 1999) prepared sucrose ester niosomes for encapsulation of ovalbumine and administered the vesicular formulations through oral route in Balb/c mice. Significant increase in antibody titers was observed following oral vaccination with less hydrophilic vesicular formulation.

x) Chattaraj and Das (Chattaraj et al., 2003) entrapped hemagglutinin antigens from three different influenza A strains in Span 40 or 60 niosomes for nasal mucosal delivery.

xi) BSA-loaded niosomes composed of Span 60/Span 85/CHOL/stearylamine were coated with a modified polysaccharide O-palmitoylmannan (OPM) for targeting them to Langerhan’s cells, the major antigen presenting cells found in abundance beneath the stratum corneum (Jain et al., 2005).

xii) Measuring serum IgG titre and its subclasses (IgG2a/IgG1 ratio) elicited a significantly higher serum IgG titre upon topical application of mannosylatedniosomes as compared with topically applied alum adsorbed BSA (P < 0.05). The mannosylatedniosomes also were used orally for induction of the oral mucosal immunization against TT (Jain et al., 2006). Coating with OPM was carried out to protect antigen encapsulated vesicles from bile salts dissolution and enzymatic degradation in the gastrointestinal tract and to enhance their affinity toward the antigen presenting cells of Peyer’s patches. On the other hand, Gupta et al. (Gupta et al., 2005) showed topically given TT containing transfersomes, after secondary immunization,
characterization of HPV candidate antigen and Development of novel vaccine delivery system could elicit immune response (anti-TT-IgG) that was equivalent to the one that produced following intramuscularly alum-adsorbed TT-based immunization. The immunity response of Span 85/CHOL niosomes was weaker than transfersomes.

xiii) Isolated virus antigens commonly have low immunogenicity; therefore there is considerable interest in designing appropriate immunological adjuvants for these vaccines. Studies of the immune responses induced by a herpes simplex type 1 (HSV-1) antigen preparation incorporated into NISV or ISCOMs, demonstrated induction of high levels of serum IgG, IgG1 and IgG2a antibodies following vaccination with both adjuvant preparations (Hassan et al. 1996). In addition, high neutralizing (NT) antibody levels were induced with both the NISV and the ISCOM HSV-1 antigen preparations and some protection against homologous and heterologous HSV challenge infection was also provided. When splenocytes from mice immunised with the adjuvant formulations were stimulated in vitro, IL-2, IL-10 and IFN-g were produced in the culture supernatants. Thus a wide range of immune activity can be elicited by HSV-1 antigens when formulated with NISV (Hassan et al. 1996) demonstrating that NISV can also successfully adjuvant antigens relevant to disease.

xiv) Toxoplasma gondii is an obligate intracellular protozoan parasite responsible for congenital infection and abortion in both man and his domestic animals. In studies using a murine model of congenital toxoplasmosis, soluble parasite antigen was entrapped in NISV and used for vaccination. Not only was the NISV based vaccine able to induce protective immunity, as measured by the reduced number of abortions and parasite burdens in this vaccinated group, but their splenocytes had enhanced production of antigen-stimulated IFN-g compared with using FCA (Roberts et al., 1994). During T. gondii infection, IFN-g -mediated protection has been primarily associated with CD8+ lymphocyte activity and therefore these results suggest that NISV are not only potent stimulators of Th1 lymphocytes, but possibly also CD8+ cytotoxic T cells.
Table 2c: Reports of niosome based vaccines

<table>
<thead>
<tr>
<th>Niosomal Composition/Type</th>
<th>Vaccine/Antigen</th>
<th>ROA</th>
<th>Inference</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose esters, cholesterol and dicetyl phosphate</td>
<td>Ovalbumin vaccine</td>
<td>Oral</td>
<td>Encapsulation of ovalbumin into Wasag®7 niosomes resulted in a significant increase in antibody titres compared to the control</td>
<td>Rentel, et al., 1999</td>
</tr>
<tr>
<td>Span 60, cholesterol and stearylamine</td>
<td>Plasmid DNA vaccine</td>
<td>Oral</td>
<td>Compared to oral, intramuscular naked DNA and recombinant HBsAg did not elicited sIgA titer in mucosal secretions that were induced by oral administration of coated niosomes</td>
<td>Jain, et al., 2005</td>
</tr>
<tr>
<td>Span 60, cholesterol, stearylamine</td>
<td>Tetanus Toxoid vaccine</td>
<td>Oral</td>
<td>Humoral as well as cellular response was elicited by the said formulation</td>
<td>Jain, et al., 2006</td>
</tr>
<tr>
<td>O-palmitoylpullulan coated niosome</td>
<td>Tetanus Toxoid vaccine</td>
<td>Oral</td>
<td>Compared to intramuscularly given alum adsorbed TT, OPP appended niosomes produced an equivalent immune response</td>
<td>Katare, et al., 2006</td>
</tr>
</tbody>
</table>

Investigations have been carried out (Brewer et al., 1996) in order to more fully characterize the immune responses generated using NISV as adjuvants using ovalbumin (OVA), as a standard antigen, and a synthetic peptide derived from measles fusion protein (Partidos and Obeid, 1992). These studies confirmed the ability of NISV to act as an adjuvant which could preferentially generate a Th1-type immune response i.e. classical CMI. This could be demonstrated by enhanced IgG2a production in vivo and antigen induced lymphocyte IL-2 generation in vitro following its use. Furthermore, it was demonstrated that NISV with entrapped antigen was able to induce MHC class I specific CTL responses. It has been suggested that the adjuvant activity of NISV, and other particulate antigen delivery systems such as liposomes, is due to their ability to target antigen to macrophages (Brewer and Alexander 1992). Significantly macrophages have been demonstrated to be the only antigen presenting cells capable of processing exogenous antigen via the endogenous pathway of class I MHC presentation (Kovacsovics Bankowski and Rock 1995).
It is thought that by interacting with cell membranes, the adjuvant is able to introduce its associated antigen directly into the cytoplasm and subsequently the endogenous antigen-processing pathway resulting in MHC class I restricted antigen presentation and CTL responses (Moreina and Lovgren-Bengtsson, 1986; Moore and Carbone, 1988). Macrophages are also major producers of IL-12, a Th1-inducing cytokine and potent inducer of cytolytic T cell activity and IL-10, which antagonises IL-12 functions (Mosmann and Moore, 1991). Ex vivo studies following injection of NISV, demonstrated an increase in synthesis of splenocyte IL-12 with a concurrent down regulation in IL-10 production. It was concluded that NISV were effective adjuvants capable of stimulating Th1 and cytotoxic T-cell responses against both protein and synthetic peptide antigens and that this activity was associated with the ability of the NISV to modulate IL-10 and IL-12 production to favor generation of Th1-type immune responses.

2.11 Cervical cancer & HPV: Cervical cancer is the fifth most common cancer in humans, the second most common cancer in women worldwide and the most common cancer cause of death in the developing countries. Sexually transmitted human papilloma virus (HPV) infection is the most important risk factor for cervical intraepithelial neoplasia and invasive cervical cancer (Schiffman et al., 2007). The worldwide incidence of cervical cancer is approximately 510,000 new cases annually, with approximately 288,000 deaths worldwide (Sankaranarayanan et al., 2006). Unlike many other cancers, cervical cancer occurs early and strikes at the productive period of a woman's life. The incidence rises in 30–34 years of age and peaks at 55–65 years, with a median age of 38 years (age 21–67 years). Estimates suggest that more than 80% of the sexually active women acquire genital HPV by 50 years of age (Singh, 2005). Hence the advent of a vaccine against HPV has stirred much excitement as well as debate.

Persistent infection with human papillomavirus (HPV) is the most important etiological factor in cervical cancer and its precursor lesions (cervical intraepithelial neoplasia, CIN), with HPV DNA being identified in more than 99% of cervical cancers. Although over 100 genotypes of HPV have been identified, only several are considered “high risk” due to their oncogenic potential, notably HPV-16 and 18.
The Human Papilloma Virus is responsible for causing cervical cancer. HPV belongs to Papillomaviruses which are small, approximately 52-55nm in diameter. HPVs are non-enveloped viruses containing a circular double-stranded DNA genome of around 8,000 base pairs, which preferentially infect squamous epithelial cells. This shape is made up of 12 pentameric and 60 hexameric capsomers arranged on a T=7 lattice. Their capsid is composed of two proteins, a major (L1) and minor (L2). HPV is part of the family known as Papovaviruses, which was named for its three main members: Papillomavirus, Polyomavirus, and simian Vacuolating Agent. They are found in many vertebrates, and exhibit high species specificity. HPV infects the basal cells of the dermal layer, and early gene expression occurs in these cells. Late gene expression and high copy DNA synthesis occurs only in terminally differentiated epidermal cells. The genome encodes at least six early genes (E1, E2, E4, E5, E6, E7) and two late genes (L1, L2). The early genes regulate viral DNA replication while the late genes encode the viral capsid for packaging newly synthesized virions. HPV infects the basal cells of the cervical epithelium through microtrauma; however, the majority of HPV infections are self-limiting and transient. In persistent infection, the expression of the HPV genome is correlated to the maturation of the infected cell.

**Figure 7:** HPV-16 genome and protein function, (J Formos Med Assoc. 2010 January; 109(1): 4–24).
HPV-16 has a 7904 base pair, double-stranded circular DNA genome. The transcriptional promoter is designated P_{97}. A_{E} and A_{L} are the early and late polyadenylation sites, respectively. Immature epithelial cells in the basal layer allow expression of the HPV early genes whereas in terminally differentiated cells, transcription shifts to the late genes, allowing the newly assembled virions to be released away from the submucosa, the site of immune surveillance. The HPV genome is usually found in episomal form in productive infection. However, high risk HPVs may integrate into the host genome in some persistent infections. This integration causes deletion of some of the early genes (E2, E4, and E5) as well as the late genes L1 and L2. E2 is a master regulator of the viral genome and notably a transcriptional repressor of the E6 and E7 genes. Loss of E2 through integration allows upregulation of E6 and E7 transcription. E6 and E7 are oncogenes, capable of inactivating tumor suppressor’s p53 and retinoblastoma (Rb), leading to genomic instability and repression of apoptosis. HPV utilizes several mechanisms to avoid and modulate the immune system, allowing HPV to freely proliferate within cells. An understanding of these defense mechanisms, HPV virology and its role in tumorigenesis has facilitated the development of preventive and therapeutic vaccines to stimulate the immune system into responding to HPV. While preventive vaccines aim to block initial HPV entry into epithelial cells, therapeutic vaccines generate a T-cell immune response to eliminate existing HPV infection and HPV-associated neoplasms.

2.11.1 Epidemiology of genital HPV infection: HPVs are a family of deoxyribonucleic acid (DNA) viruses that infect skin or mucosal epithelial cells. Of the more than 100 types of HPV, at least 13 can cause cancer of the cervix, other anogenital organs, or the head and neck. Other HPV types, with low oncogenic potential, cause non-malignant lesions, such as anogenital warts. Genital HPVs are highly transmissible, and infection with HPV is the most common viral infection of the genital tract. Infection is extremely common throughout the world. Genital HPV types are transmitted through penetrative and non-penetrative sexual contact. Because HPV is usually acquired within the first few years after onset of sexual activity, peak incidence usually occurs between the ages of 16 and 20 years. Incidence and prevalence increase with increasing sexual activity. Most people will acquire the infection at some time in their life. Risk factors for transmission of HPV infections include multiple sex
partners, lack of condom use, smoking and coinfection with other sexually transmitted infections (STIs), including human immunodeficiency virus (HIV). HPV is a purely mucosal infection and has no bloodstream phase. Only about half of women develop serum antibodies after natural infection, and antibodies do not necessarily prevent subsequent infection. Most HPV infections of the cervix are asymptomatic and transient; most clear within 2 years (i.e. are no longer detectable by commonly used molecular methods). The small proportion of HPV infections that persist can cause neoplastic changes.

**2.11.2 Role of HPV infection in malignant and non-malignant diseases:** Persistent HPV infections can lead to development of precancerous lesions, cancer, notably cervical cancer, and non-malignant disease. Persistent cervical infection causes cellular changes in the epithelium that can be detected through cytology screening. Persistent infection can cause cervical intraepithelial neoplasia (CIN), which is graded as CIN1, CIN2 or CIN3, according to the extent of affected epithelium, and adenocarcinoma in situ (AIS). Moderate to high-grade CIN (CIN2/3) and AIS – diagnoses confirmed by cervical biopsy – have a high probability of progression to cancer and are considered precancerous lesions. The time between initial HPV infection and development of cervical cancer is usually decades.

High-grade anal, vaginal and vulvar intraepithelial neoplasia have a high probability of progressing to cancer. Several factors contribute to HPV persistence and development of cervical cancer: older age immune suppression due to HIV or other factors, multiparity, early age at first delivery, long term, hormonal contraceptive use, smoking and infection with other STIs. Oncogenic types of HPV are estimated to cause 100% of cervical cancers; 90% of anal cancers; 40% of cancers of the vulva, vagina, and penis; and at least 12% of head and neck cancers. HPV 16 and 18 cause approximately 70% of cervical cancer cases globally. Together, HPV 16, 18, 31, 33, 35, 45, 52 and 58 account for about 90% of cervical cancer cases in all regions. HPV 16 is the most common cause of cervical cancer, causing 52–58% of cases in all regions. HPV 16 is the most common cause of non-cervical anogenital cancers.

Together, HPV 6, 11, 16 and 18 cause about 35–50% of CIN1, vulvar intraepithelial neoplasia (VIN) 1 and vaginal intraepithelial neoplasia (VaIN)1 cases. HPV types 6 and 11, two types with very low oncogenic potential, cause 90–100% of external anogenital warts, and nearly all cases of recurrent respiratory papillomatosis (RRP). Cervical cancer is the
most common HPV-related malignancy. It is the leading cause of cancer among women in developing countries, and the second leading cancer in women worldwide. In 2005, nearly 500,000 new cases of cervical cancer occurred. If current incidence trends continue, incidence of cervical cancer will rise to an estimated 1 million cases per year by 2050.

Incidence rates are highest in parts of Latin America and the Caribbean, sub-Saharan Africa, Melanesia and parts of south Asia. In 2002, 54% of cervical cancer cases occurred in Global Alliance for Vaccines and Immunization (GAVI)-eligible countries, of which about half were in India alone. Incidence rates range from less than 1–50 per 100,000. In 2005, more than 260,000 cervical cancer deaths occurred, resulting in 2.7 million years of life lost. If current trends persist, cervical cancer deaths are expected to rise by nearly 25% in the next 10 years. About 80% of cervical cancer deaths occur in developing countries; if current mortality trends continue, this proportion is expected to increase to 90% by 2020. In most developing countries, more than 60% of women with cervical cancer will die of their disease due to late detection. Incidence and mortality of cervical cancer is highest in countries where effective screening, diagnosis and treatment services are absent or limited. In the Caribbean, Eastern Europe and Latin America, cervical cancer contributes more to years of life lost than tuberculosis, maternal conditions or acquired immune deficiency syndrome. Most cervical cancer cases occur in women aged over 40 years, an age when women maximize their familial, economic, social, and educational contributions, including workforce and community participation, child and elder care, and support of child education.

The HIV pandemic has increased the global cervical cancer burden; HIV-infected females are more likely to acquire oncogenic HPV types that progress rapidly to neoplasia.

Cancers of the vulva, vagina, penis, anus, and head and neck due to HPV are far less common than cervical cancer; age-standardized incidence rates are less than 2 per 100,000. Most of these HPV-related anogenital and head and neck cancer cases occur in adults aged over 50 years. Incidence of HPV-related vulvar, anal, and head and neck cancer is rising in some regions or subpopulations.

2.11.3 Indian Scenario of HPV Infection: Cervical cancer is ranked as the most frequent cancer in women in India. India has a population of approximately 365.71 million women above 15 years of age, who are at risk of developing cervical cancer. The current estimates
indicate approximately 132,000 new cases diagnosed and 74,000 deaths annually in India, accounting to nearly 1/3rd of the global cervical cancer deaths. [WHO information] Indian women face a 2.5% cumulative lifetime risk and 1.4% cumulative death risk from cervical cancer. At any given time, about 6.6% of women in the general population are estimated to harbor cervical HPV infection. HPV serotypes 16 and 18 account for nearly 76.7% of cervical cancer in India. Warts have been reported in 2–25% of sexually transmitted disease clinic attendees in India; however, there is no data on the burden of anogenital warts in the general community. There are currently several cervical cancer research programs in India. The National cancer registry program established by the Indian council of medical research acts as a surveillance system for cancer in India. It collects data in an “active” manner, visiting government and private sector hospitals, specialized cancer hospitals and pathology laboratories to get information on the types and magnitude of cancer cases. The cancer registry in India does not cover the entire country actively but collects information only from a few urban and rural registries established in the country.

a. Burden of non-malignant disease related to HPV 6 and 11: Anogenital warts are common among sexually active persons. Global burden estimates are imprecise, but some surveys indicate that up to 10% of women have had anogenital warts. Warts are highly infectious; they generally appear in adolescence or young adulthood shortly after onset of sexual activity, usually in the late teens and 20s. Warts may cause pain, bleeding, genital or urethral obstruction, pregnancy complications, shame and embarrassment. Warts infrequently resolve without treatment, and recurrence is common despite treatment. In HIV-infected or immunocompromised persons, warts may be severe and require inpatient treatment. Warts incur substantial health-care costs, especially in high-income countries that routinely provide wart diagnosis and treatment. RRP is a rare but serious condition of the larynx. In children, it results from perinatal exposure during delivery, while in adults it results from oral–genital sexual contact. RRP often requires repeated surgical treatment, especially if the airway is compromised.

The sero-conversion rates for Cervarix and Gardasil is 97.5% or higher in women. The antibody response generated is unfortunately type-restricted to those HPV genotypes contained within the vaccine. However, there is some low-level cross-protection against
other closely related genotypes. Despite this partial cross-protection, a preventive vaccine would need to contain the eight most common HPV types found in cancer to create >90% protection against cervical cancer – a costly and complex process. Ongoing studies show continued protection for up to 6.4 years post vaccination with HPV-16 and 18 L1 VLPs, as well as some cross-protection to HPV 45 and HPV 31. One monovalent HPV-16 L1 vaccine with an aluminiumhydroxyphosphate sulfate adjuvant shows 86% of volunteers are seropositive at an average 8.5 years, where mean HPV-16 antibody titers were 71.7 mMU/mL (milli-Merk units [mMU]/mL) in contrast to 150 mMU/mL at 4 years.

**Table 2d: Comparison of Cervarix and Gardasil preventive HPV vaccines**

<table>
<thead>
<tr>
<th></th>
<th>Cervarix</th>
<th>Gardasil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Merck &amp; Co</td>
<td>GlaxoSmithKline</td>
</tr>
<tr>
<td>HPV types</td>
<td>16,18</td>
<td>16,18,6,11</td>
</tr>
</tbody>
</table>
| Antigen / dose   | 20 µg of HPV 16L1  
20 µg of HPV 18L1 | 40 µg of HPV 16L1  
20 µg of HPV 18L1  
20 µg of HPV 6L1  
20 µg of HPV 11L1 |
| Antigen source   | Baculovirus                       | Yeast                             |
| Adjuvant         | AS04 composed of: 500 µgaluminium hydroxide  
50 µg MPL (3-O-desacyl-4'-monophosphoryl lipid A) | 225 µg aluminum hydroxyphosphate sulfate |
| Recommended administration | 0.5 mL dose at 0, 1, 6 months intramuscular dose | 0.5 mL dose at 0, 2, 6 months intramuscular dose |
| Approx. price    | Rs. 5000/dose                     | Rs. 6000/dose                     |
| Approved for ages| 10 – 25                           | 9 - 26                            |
| Antibody titers 1 month after completed vaccination course compared to natural infection | HPV 16: 107 TIMES  
HPV 18: 82 TIMES | HPV 16: 105 TIMES  
HPV 18: 19 TIMES  
HPV 6: 11 TIMES  
HPV 11: 7 TIMES |
| Geometric mean antibody titers at 7 months (age 18–26) | HPV 16: 31715  
HPV 18: 13732 | HPV 16: 8682  
HPV 18: 1886 |
2.11.4 HPV Vaccination in Males: HPV vaccine is not licensed for use among males. Efficacy studies among males are under way. Australia is the first country to approve the quadrivalent HPV vaccine in males (between 9 and 15 years old), and the vaccine was approved for administration to males between the ages of 9 and 26 years in other developed nations.(Schiller JT et al., 2008)

2.11.5 Therapeutic HPV vaccines development: Several factors highlight the need for a therapeutic, rather than preventive, vaccine. The most pressing of these factors is the high prevalence of existing HPV infection worldwide, on which preventive vaccines make little impact. Since over 80% of cervical cancer cases occur in the developing world, preventive vaccines would need to be in widespread use for many years to reduce this figure, which is currently improbable in the near future due to logistics and cost. Furthermore, in HPV-associated malignancies where genomic integration has occurred, infected cells may no longer express L1 or L2. To exert a therapeutic effect, a different vaccine target antigen is needed which is expressed constitutively in HPV-associated tumor cells. Such a vaccine may exert an immediate effect on the mortality and morbidity of HPV-associated lesions.

<table>
<thead>
<tr>
<th>Table 2e: Therapeutic HPV vaccine advantages and disadvantages and future strategies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peptide-based</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Protein-based</strong></td>
</tr>
<tr>
<td><strong>DNA-based</strong></td>
</tr>
<tr>
<td><strong>RNA-based</strong></td>
</tr>
</tbody>
</table>
The HPV E6 and E7 antigens represent ideal targets for therapeutic vaccines since these are constitutively expressed in HPV-infected cells and not healthy cells. E6 and E7 are essential to the induction and maintenance of cellular transformation, and thus are unlikely to be lost in an attempt to evade the immune system. A number of therapeutic vaccines have been developed targeting E6 and E7 including live vector vaccines, peptide/protein-based vaccines, cell-based vaccines and nucleic acid-based vaccines, each with advantages and disadvantages. These vaccines likely HPV infection through cell-mediated immunity (Figure 8) and many have shown promise in both preclinical and clinical trials.

Figure 8: Therapeutic HPV vaccines (J Formos Med Assoc. 2010 January; 109(1): 4–24).

A number of therapeutic vaccines have been developed targeting HPV E6 and/or E7 antigen(s), including live vector-based vaccines, peptide/protein-based vaccines, nucleic acid-based vaccines and cell-based vaccines. These vaccines likely to simulate HPV infection through cell-mediated immunity, Dendritic cells (DCs) prime naïve T cells through MHC: Antigen (Ag) complex with the help of costimulatory molecules (B7 on the DC and CD28 on...
Characterization of HPV candidate antigen and Development of novel vaccine delivery system

the T cell). Antigens are processed and presented to CD4+ T cells via MHC class II pathway and presented to CD8+ T cells via MHC class I pathway. The primed effector T cells are subsequently HPV-antigen-specific T cells. Activated CD8+ T cells kill tumor cells by inducing apoptosis in the target cells. Induction of CD4+ T cell help can augment the CD8+ T cell immune response, supplementing tumor killing.

2.12 HPV & Mucosal immunization:

i) A study conducted by Dominique Fraillery et.al., (2009), on rectal and vaginal immunization of mice with human papilloma virus L1 virus like particles provide significant evidence that mucosal immune response is of paramount important in combating infections such as HPV. As detailed in the literature review, Human Papilloma virus (HPV) vaccines based on L1 virus like particle (VLP) can prevent genital HPV infection and associated lesions after three intramuscular injections. Needle free administration might facilitate vaccine implementation of HPV16L1 VLPs in mice and their ability to induce anti-VLP and HPV-16 neutralizing antibodies in serum and genital, rectal and oral secretions. The study also demonstrated that rectal and vaginal immunizations were not effective in the absence of the adjuvant. Cholera toxin was able to enhance systemic and mucosal anti-VLPs responses in after rectal immunization.

ii) A study conducted by Nardelli-Haefliger et al., (2005), women were vaccinated with escalating doses of HPV16L1 VLPs via nasal nebulisation, bronchial aerosilisation, or a combination of intramuscular and aerosol vaccination. The alternative routes of vaccination were well tolerated and many of the volunteers who received aerosol vaccinations exhibited serum antibody titers that were comparable to those induced by intramuscular vaccination. A mucosal immune response was induced by aerosol vaccination as demonstrated by the induction of anti-HPV16 VLP SIgA in secretions.

Hence, this research involved the formulation of HPV16 L1 VLP using Niosomes as vaccine carriers with and studied the immune response in systemic and mucosal system with immunizing through both subcutaneous & intranasal route.
3.1. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

Purpose: SDS PAGE was used for the characterization of HPV L1 Protein under different physicochemical conditions.

Equipment/Instruments
- BIO-RAD Mini-PROTEAN Tetra cell
- Power pack
- Micropipettes
- Rotating shaker
- Water bath (95 + 5oC)

Materials
- Acrylamide 30% w/v
- Tris-HCl-1.5M, pH 8.8
- Tris-HCl-0.5M, pH 6.8
- Ammonium persulphate 10% w/v (APS)
- Sodium dodecyl sulphate 10% w/v (SDS)
- Tetramethylethylenediamine (TEMED)
- Running buffer, pH 8.3 (10X)
- Loading buffer (5X)
- Methanol
- Acetic acid
- Molecular weight markers
- Coomassie brilliant blue (R 250)

PREPARATION OF BUFFERS AND REAGENTS

Running buffer (10X): Weigh 30.28 g of tris (hydroxymethyl) methylamine, 144.2 g of glycine, and 10 g of sodium dodecyl sulphate. Dissolve and make up to 1000 mL with WFI. The pH of the diluted solution should be between 8.1 and 8.8; this solution can be stored at room temperature for three months.

Running buffer (1X): Take 100 mL of above prepared 10X running buffer and dilute it to 1000 mL with WFI. Freshly prepare the solution before use.
Sample loading buffer concentrated

**Reducing buffer (5X)**

<table>
<thead>
<tr>
<th>Buffer components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (hydroxymethyl)methylamine</td>
<td>3.78 g</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>10 g</td>
</tr>
<tr>
<td>bromophenol blue</td>
<td>100 mg</td>
</tr>
<tr>
<td>Glycerol</td>
<td>50 mL</td>
</tr>
<tr>
<td>Dissolve and makeup to 200 mL with WFI</td>
<td></td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>25 mL</td>
</tr>
</tbody>
</table>

Adjust the pH to 6.8 with hydrochloric acid and makeup to 250 mL with WFI. This solution can be aliquoted in micro centrifuge tubes and stored at -20°C for three months.

**Note:** In place of 2-mercaptoethanol, dithiothreitol (DTT) may also be used as a reducing agent. In this case add DTT just before use to final concentration of 100 mM.

**Non-reducing buffer (5X)**

<table>
<thead>
<tr>
<th>Buffer components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (hydroxymethyl)methylamine</td>
<td>3.78 g</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>10 g</td>
</tr>
<tr>
<td>bromophenol blue</td>
<td>100 mg</td>
</tr>
<tr>
<td>Glycerol</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

Dissolve the contents in 100 mL of WFI. Adjust the pH to 6.8 with hydrochloric acid and makeup to 250 mL with WFI. This solution can be aliquoted in micro centrifuge tubes and stored at -20°C for three months.

**Acrylamide 30%**

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>29.0 g</td>
</tr>
<tr>
<td>Bisacrylamide</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

Dissolve and makeup the solution to 100 mL with WFI. This solution can be stored at 2 to 8°C for three months.

**Tris-HCl,1.5M (pH 8.8):** Dissolve 90.8 g of tris (hydroxymethyl) methylamine in 400 mL of WFI. Adjust the pH to 8.8 with hydrochloric acid and make up to 500 mL with WFI. This solution can be stored at room temperature for three months.