CHAPTER II
LITERATURE REVIEW

Japanese Encephalitis (JE)

Japanese encephalitis (JE) is one of the most important endemic encephalitis in the world caused by JE virus which is one of 70 viruses in *Flavivirus* genus of the Flaviviridae family. The complete genomic sequences of JE virus and several other flaviviruses have already been determined, including yellow fever (YF) virus, the prototype virus in the family. Morphologically, flaviviruses are spherical, approximately 40 to 50 nm in diameter, with a lipid membrane enclosing an isomeric 30 nm diameter nucleocapsid core comprising a capsid (C) protein and a single strand messenger (positive) sense viral RNA (Misra and Kalita, 2010; Appaiahgari et al., 2005; Wu, Huang and Tao, 2003). Membrane surface projections are composed of glycosylated envelope (E) and membrane (M) protein, a mature form of the pre-membrane (prM) protein as shown in Fig 2.1 (Halstead and Tsai, 2004; Yang et al., 2004; Solomon et al., 2003; Lindenbach and Rice, 2001).

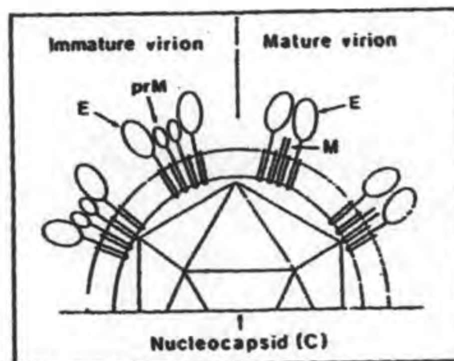

Figure 2.1 Morphology of Flaviviruses cell membrane (Lindenbach and Rice, 2001)

The order of proteins encoded in the JE virus of opening reading frame (ORF) region, as with other flaviviruses, is 5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. Among the surface proteins of JE virus as shown in Fig 2.2, the prM,

หอสมุดกลาง สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

E, NS1-NS2A-NS2B-NS3and NS5 could be used to stimulate the humural as well as the cell-mediated immunity of JE (Konishi et al., 2000; Ashok and Rangarajan, 2000).

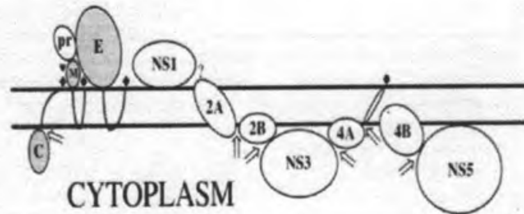

Figure 2.2 The surface protein of JE virus (Lindenbach and Rice, 2001)

Flaviviruses replicate in a variety of cultured cells of vertebrate and arthropod origin. Viral entry occurs by receptor-mediated endocytosis, with the formation of coated vesicles, or by direct fusion with plasma membrane. The nucleocapsid is uncoated by acid-dependent fusion of viral and endosomal membranes, releasing genomic RNA into the cytoplasm where viral replication continues with immediate translation of the uncoated viral genome (Knipe et al., 2001). The translated polyprotein then is processed and assembled into a virus-specific replication complex (Fig. 2.3).

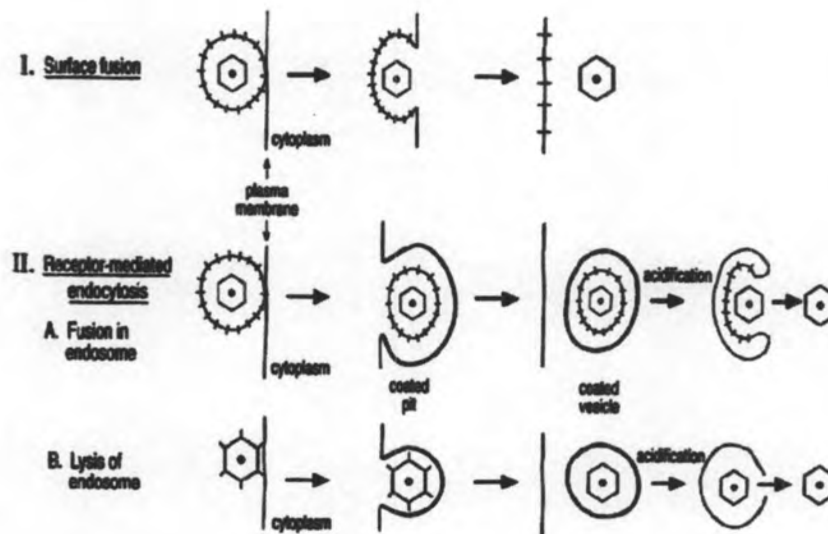

Figure 2.3 The cell entry of virus (Knipe et al., 2001)

The hydrophobic carboxyl terminus of E protein provides a membrane associated anchor, while an extensive ectodomain, stabilized by disulfide bridging, is folded into three antigenic domain (A,B,C) as shown in Fig 2.4 that are variably related to (1) determinants representing flaviviral group, sub-group, virus specific epitopes and (2) biological function. Crystallographic examination of the viral E protein reveals that the structure was a homodimer fold into three distinct structural domains I, II and III corresponding to the antigenic domains C, A and B, respectively. Domain III contains an immunoglobulin-like module extending perpendicular to the viral surface that is likely to be involved in receptor binding. Binding of JE virions to certain cells of CNS lineage may be associated with the presence of specific neurotransmitter receptors. Dengue-2 virus selectively binds cellular heparin sulfates of the glycosaminoglycan (GAG) family via E protein GAG-binding motifs within the carboxyl terminal and externally accessible regions of domain I and III. Similar mechanisms may apply to JE and other flaviviruses (Wu, Huang and Tao, 2006; Kaur, Rauthan and Vrati, 2004; Wu et al., 2004; Chang, Derek and Springfield, 2003 and Kolaskar and Kale, 1999).

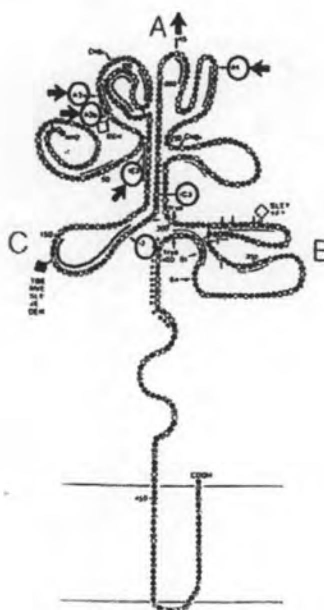

Figure 2.4 Domain A, B and C of Japanese encephalitis virus (Ivy, Nakano and Clements, 2000)

Pathogenesis of JE

After an infectious mosquito bite, viral replication occurs locally in regional lymph nodes. Virions disseminate to secondary sites, where further replication contributes to a viremia. Invasion of the CNS probably occurs from the blood by antipodal transport of virions through vascular endothelial cells. Infection in the CNS spreads by viral dissemination through the extracellular space or by direct intercellular spreads. Sensitized T-helper cells (Th) stimulate an inflammatory response by recruiting macrophages and lymphocytes to the perivascular space, parenchyma and CNS, where the inflammatory response clears infected neurons, with subsequent formation of glial nodules. The predominant cell type in the CSF and in the parenchyma is helper/inducer ($CD4^+$) T cells, with B lymphocytes confined chiefly to the perivascular space (Wu et al., 2003; Konishi et al., 2003 and Ramakrishna et al., 1999).

Immunization of JE

Three JE control strategies have been considered based on transmission cycle of JE including vector control, swine immunization and immunization of humans. The control of mosquito, JE vector, apparently decrease vector density but the effect is just a moment. However, environment and socio-agricultural background support sample vector breeding site. Although, immunization of swine is carried out in several regions, many problems have been recognized. Therefore, human immunization remains the most reliable control for human JE at present (Halstead and Tsai, 2004).

Successive refinements of the mouse brain vaccine were introduced by research institutes in Japan, leading to the current purified Japanese encephalitis vaccine. Mouse brain-derived vaccines are produced in Japan and elsewhere using a similar sequence of centrifugation, ultrafiltration, protamine sulfate precipitation, and formalin inactivation in the cold, followed by further purification by ultrafiltration, ammonium sulfate precipitation, and continuous rate zonal centrifugation on sucrose density gradients (Halstead and Tsai, 2004; Flamand et al., 1995).

Minimal immunogenicity and potency in mice (compare with a vaccine standard) and maximal total protein (80μg/ml) and myelin basic protein (MBP) content (2ng/ml) are the specifications of vaccine. Bulk vaccine is diluted with medium 199 and phosphate buffer to meet a potency standard. Although the quantity of JE, E protein is not controlled, in one study, a dose was estimated to contain approximately 50 μg. The vaccine is stabilized with gelatin and sodium glutamate and preserved with thimerosol. In Japan, the vaccine is distributed principally in liquid form; for international distribution, it is lyophilized and reconstituted with sterile water. Lyophilized vaccine is stable at 4^0C for at least 1 year and retains more than 90% of its potency after 28 weeks at 22^0C. At 37^0C, lyophilized vaccine retains 95% of its original potency after 4 weeks. After reconstitution, a JE vaccine is stable at 22^0C for at least 2 weeks, but at 37^0C, potency declines to 85% (Misra and Kalita, 2010; Halstead and Tsai, 2004).

Mucosal immune system

Mucosal surfaces, such as the gastrointestinal tract, respiratory and genital tracts, are the principal sites of entry and colonization for many pathogens. In order to effectively protect these surfaces and to stimulate the systemic immune response, the mucosal immune system needs to be properly activated through mucosal immunization (Chang, Derek and Springfield, 2003). The antibodies on mucosal surfaces and in blood circulation are the results of the local exposure of antigens to the mucosal-associated lymphoid tissues (M-cells), especially those in the upper respiratory tract (nasal lymphoid tissue, or NALT), and the gastrointestinal tract (gut associated lymphoid tissue, or GALT). Anatomically, the GALT consists of the Peyer's patches, the appendix, and solitary lymph nodes while the NALT contains the palatine, lingual and the pharyngeal tonsils as lymphoid tissues. The epithelial surfaces of both the NALT and GALT contain specialized antigen–sampling cells known as M-cells. These cells can transport antigens from the mucosal surfaces into the underlying lymphoid tissues (Ivy, Nakano and Clements, 2000; Jung et al., 2000 and McGhee et al., 1992).

The subepithelial regions of mucosal surfaces of gastrointestinal tract, respiratory tract and genital tracts contain an abundance of immunocomponent cells

such as antigen presenting cells (APC; MHC class II-positives) T and B cells in lymphoid tissue. These T cells, B cells and APC are organized into mucosal-associated lymphoid aggregates (follicles), which are the main components of the mucosal immune inductive site. After entering into the MALT, the antigens are rapidly internalized and processed by antigen presenting cells such as subepithelial dendritic cells, sampling B-cells and macrophages in the follicle, and presented to T cells and B cells located in the lymphoid tissue. Upon sensitization by antigens, B cells proliferate and switch to immunoglobulin committed cells (Ig cells), especially immunoglobulin A (IgA) which are usually produced at mucosal sites. These immunoglobulin committed cells eventually leave the mucosal lymphoid follicle (inductive site) and migrate through the systemic circulation to various mucosal lymphoid effector site such as lamina propia, for terminal differentiation to secretory immunoglobulin producing plasma cells (Wu et al., 2003; McGhee et al., 1992).

The mucosal immune system differs in several other ways from the systemic immune system. Mucosal immune system frequently results in the stimulation of both mucosal and systemic immune responses, while systemic immune system typically only induces systemic responses without activation of the mucosal immune system. Induction of mucosal responses leads to production of secretory IgA (sIgA) antibodies, which are not usually produced by systemic immunization (Ramakrishna et al., 1999).

M-cells and nasal cells

According to M-cells, M-cells are differed from nasal cells in many ways. There is a loss of brush border organization that manifested as a lack of apical membranous villi, a feature that is exemplified by re-distribution of cytoskeletal protein villin from the apical surface to cytoplasm (Tyrer et al., 2002; Kerneis et al., 1996) as shown in Fig 2.5. Digestive function also disappears as indicated by the loss of brush border enzymes such as alkaline phosphatase (Tyrer et al., 2002; Brown et al., 1990). The cell adhesion molecule, $\alpha 5\beta 1$integrin exhibits a different distribution pattern with M-cells expressing on apical surfaces while nasal cells exhibiting on the basolateral sites (Tyrer et al., 2002; Clark, Hirst and Jepson, 1998).

Nasal cells are the type of the mucosal cells composed of various cell types. The vestibular surface is covered with stratified squamous epithelim where short stiff hairs filter the large particles from incoming airstream. The respiratory area is covered with pseudostratified columnar epithelium cells. The olfactory region comprises a small patch of columnar cells containing the smell receptor. Particularly important for nasal drug absorption is the respiratory epithelium which is the pseudostratified columnar epithelium. These epithelium consist of four types of columnar cells : columnar cells with and without cilia, mucus containing goblet cells and basal cells. The thickness of respiratory epithelium is approximately 100 μm (Merkus and Verhoef, 1997; Brayden and Baird, 2001). The apical membrane of cells compose of villi and villin protein. The basolateral membrane express the cell adhesion protein α5β1. The respiratory epithelium is covered with a mucus layer. Glycoproteins are responsible for this gel like structure, mucus layer. There are plenty amount of enzyme within the mucus layer, mostly are the alkaline phosphatase enzyme (Mackay, Williamson and Hastewell, 1991).

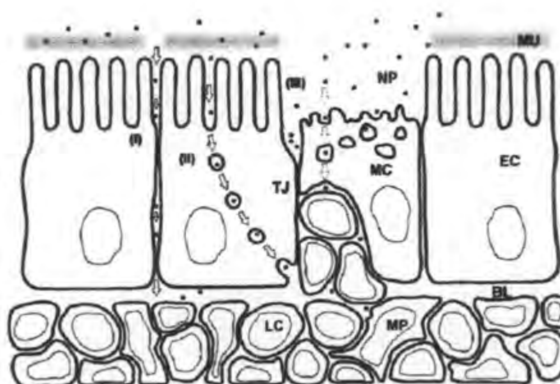

Figure 2.5 Schematic drawing of mucus (MU) covered absorptive enterocytes (EC) and M cells (MC), lymphocytes (LC) and macrophages (MP) from underlying lymphoid tissue can pass the basal lamina (BL) and reach the epithelial cell layer which is sealed by tight junctions (TJ) (Jung et al., 2000).

Effective stimulation of mucosal immune system requires the fulfillment of the following two requirements. First, vaccine antigens need to be delivered efficiently to mucosal inductive lymphoid tissues. Second, immune responses induced within the lymphoid tissues need to be enhanced through co-administration of

appropriate vehicles and adjuvants. According to the antigen administered via mucosal route, the delivery vehicles not only help the antigen to transport via M-cells, but also have to protect the antigen from enzymatic degradation. Recent advances in vaccine delivery system such as particles would be an alternative option (Nugent, Pol and Scott, 1998).

Role of particles as mucosal antigen delivery

Various physiological barriers on the mucosal surfaces prevent efficient absorption of mucosal delivered vaccines into lymphoid tissues. These barriers include enzymatic degradation of antigens, mechanical clearance of antigens from the mucosal surfaces, and low uptake efficiency of antigens by M-cells. Of the many important roles of this barrier, it is the distinct responsibility of the mucosal immune system to sample and discriminate between harmful and beneficial antigens (Acheson., 2004; Jung et al., 2000; Karlsson, Wikman and Artursson, 1993). As a result of mucosal barrier, a large antigen dose with multiple administration is usually required for induction of immune responses on the mucosal surfaces (Chen, 2000; O' Hagan, Mackichan and Singh, 2001). Regarding to the protection of antigen from nasal barriers and to reduce the dose and frequency of vaccine administration, effective delivery vehicles such as particulate carrier with the suitable size for M-cells uptaking and the aid of mucoadhesive substances to assist the adhesion to mucosal surface could be considered. Microencapsulation involves the coating of a bioactive agent, such as a vaccine, in a protective wall material which is polymeric in nature to protect the bioactive agent from mucosal degradation. Furthermore, the microsphere is a spherical particles which can be produced in a size range from ~< l um to as large as 3 mm in diameter. The particular system of interest for studies with vaccines involves the use of poly(D,L-lactide-co-glycolide) (DL-PLG) copolymers. These biocompatible polyesters are currently used in resorbable sutures and biodegrade *in vivo* into lactic and glycolic acids through the hydrolysis of ester linkages (McGhee et al., 1992). Jaganathan and Vyas (2006) demonstrated the ability of PLGA microspheres with chitosan using hepatitis B as a model vaccine and found that particles could induced strong systemic and mucosal immunity. Many groups also studied the effect of charged along with the hydrophilic-hydrophobic properties of

coating material as delivery vehicles such as, PLGA with stearylamine and polyethylenimine (Thomas, Gupta and Ahsan, 2009) PLGA with alum (Kanchan, Katare and Panda, 2009) and PLGA with PEG (Zhiqin, 2007). PLGA with carbopol and pluronic (Zou et al., 2008). Though, the use of polymer with mucoadhesive substances as particulate delivery system could entirely express advantages of each polymer and protecting the entrapped antigen from mucosal barrier as well as facilitate the cell attachment. Nowadays, mucosal vaccine is an alternative option to replace an old fashion vaccine with the aid of vaccine delivery particles. The effective vaccine delivery particle usually constructed from the biodegradable polymeric particles with the support of mucoadhesive substances.

Biodegradable polymeric particles

Micro-and nano-particles made from biodegradable polymers have been shown to effectively encapsulate some vaccine antigens and protect them from mucosal degradation. The main advantage of these polymers is their biodegradability. Polymer degradation rate can be tailored to release antigens over an extended period of time and thus reduces the frequency of vaccination to establish long term immunity (Csaba, Sanchez and Alonso, 2006). Many different types of biodegradable polymers have been studied for vaccine delivery. Among them, poly (D,L-lactic-co-glycolic acid) (PLGA) polymer is studied most extensively due to its long and safe history of human use as absorbable sutures. The PLGA polymer is biodegradable through hydrolysis to give endogenous metabolites lactic and glycolic acids. The release rate of an encapsulated antigen from PLGA particles is controlled by particle degradation rate, which is in turn determined by the polymer composition and its molecular weight. Studies in various animals have demonstrated the potential of PLGA particles as mucosal vaccine carriers. Immunized animals also showed protection against live bacterial challenges (Wei et al., 2004; Esperaza and Kissel, 1992 and Raghuvanshi et al., 2002).

Mucoadhesive substances

Mucoadhesion is where two surfaces, one of which is a mucous membrane, adhere to each other. Mucoadhesive materials are hydrophilic macromolecules containing numerous hydrogen bond forming groups. The mechanism by which mucoadhesion takes place has been said to have two stages, the contact stage followed by the consolidation stage (the establishment of the adhesive interactions). The relative importance of each stage depends on the individual application. For example, adsorption is a key stage if the dosage form cannot be applied directly to the mucosa of interest, while consolidation is important if the formulation is exposed to significant dislodging stresses. New mucoadhesive materials with optimal adhesive properties are now being developed, and these should enhance the potential applications of this technology (Smart, 2005).

Chitosan is one of a hydrophilic-biocompatible polymer studied in various animals and originated the potential to stimulate an immune response of vaccine (Agrawal, Strijkers and Nicolay, 2010). The mucoadhesive properties of chitosan and chitosan microspheres were evaluated by studying the interaction between mucin and chitosan in aqueous solution and discovered that a strong interaction was detected. A salt-bridge effect has been proposed for the interaction of the positively charged mucoadhesive chitosan microspheres with the negatively charged mucus glycoprotein (He, Davis and Illum, 1998 and He, Davis and Illum, 1999). Thus, the mucoadhesive properties of chitosan was designed and studied by increasing formulation retention prolonging in consequence to drug mucosa contact time (Perioli et al., 2008). Lubbeb et al. (2003) also found that chitosan could enhance both systemic and local immune response against diphtheria toxoid after oral and nasal administration in mice.

Aluminium in the form of aluminium hydroxide continues to be commonly used as an adjuvant in vaccines and is the only adjuvant licensed for use in human according to US perspective (Baylor, Egan and Richman, 2002; Skea and Barber, 1993). The major advantage of using aluminium adjuvants is the more rapid development of high tittered and long lasting antibody response after primary immunization. Aluminium hydroxide has been identified as crystalline aluminium oxyhydroxide with a structure of the mineral boehmite. It has high surface area with an isoelectric point of 11 that is positively charged at physiological pH (Linblad,

2004; Baylor, Egan and Richman, 2002; Matheis et al., 2002 and Gupta, 1995). Thus, the electrostatic interaction could be expected between positively charged aluminium hydroxide and negatively charged mucus glycoprotein and phosphate group of cell membrane. Katare and Panda (2006) observed the immunogenicity of mice immunized with particles co-administered with alum and found that admixture of alum and particles improved and enhanced the overall antibody response and more of Th2 type response. Moreover, HogenEsch (2002) reported that aluminium compounds could further enhance the immune response by directly stimulation of dendritic cells.

Mucosal transportation

According to particles when administered mucosally, the microspheres protect the encapsulated antigen from proteolytic digestion and those of ~< 10 μm are readily taken up by the M cells and transported into the T- and B-cell zones and which provide a sustained release of antigen (McGhee et al., 1992) while those of the smaller size are taken up by normal mucosal cell. The surface properties of the particles were identified as crucial for the success of the delivery and it has already been demonstrated that positively charged particles were more absorbed than and transport to mucosal associated lymphoid cell than the neutral or negatively ones. This could be explained by an electrostatic interaction between the cationic particle surface and anionic structures (Rieux et al., 2005). There is still much controversy surrounding the mechanism of particle transport across the epithelial barrier. For the transport of particulates, different pathways can be distinguished (Jung et al., 2000).

The paracellular route

Since the paracellular spaces, sealed by tight junctions, contribute less than 1% of the mucosal surface area and the pore diameter of these junctions was reported to be approximately at 10 A°, significant paracellular transport of macromolecules and particles is an unlikely event. The absorption enhancement of Karlsson et al. (1999) study demonstrated in the in vitro models agrees with results obtained in vivo, supporting the conclusion that a more pronounced disruption of the tight junction barrier than that obtained through stimulation of epithelial absorption of water is

required for efficient enhancement of paracellular absorption (Hochman and Artursson, 1994). Thus, delivery through paracellular route mainly concerned with disruption of tight junction. Paracellular permeability for peptides can be enhanced, however, by polymers, such as chitosan by charge mediated mechanism resulting in the binding of particles to epithelia, by a structural reorganization of tight junction-associated proteins (chitosan) (Jung et al., 2000; Issa et al., 2009).

The endocytotic route

Endocytotic processes are characterized by pinching of membrane vesicles from the plasma membrane, followed by an internalization of the engulfed extracellular materials. The endocytic pathway is controlled by a series of highly complicated and iterative molecular sorting events which result in the transport of membrane vesicles to subcellular compartments. Of particular interest are transcytotic processes at mucosal surfaces by which macromolecules or particles, internalized at the apical plasma membrane of the epithelial cells, are transported to the contralateral plasma membrane and released to the basolateral compartment. Receptor mediated endocytosis (RME) is an active transport mechanism, requiring receptors at the apical cell membrane, by contrast, adsorptive endocytosis does not require specific receptor interactions at the cells surface. The adsorptive endocytosis is initiated by an unspecific physical adsorption of material to the cell surface by electrostatic forces and is followed by an invagination of the local plasma membrane, forming intracellular vesicles. This process occurs predominantly at the base of microvilli. Adsorptive endocytosis is energy-dependent, saturable and can lead to either intracellular processing or transcytotic transport of the engulfed macromolecules or particles. The particle transport was size-dependent and micron-size could also be transported (Rieux et al., 2005; Gullberg et al., 2000 and Jung et al., 2000). Since specific receptors are not required, adsorptive endocytosis depends primarily on size and surface properties of the adsorbed material. Therefore, this mechanism may have the potential for mucosal delivery of macromolecules and colloidal particles (Jung et al., 2000).

Role of particles as in vivo mucosal stimulation

Particles systems may also be used to stimulate long lasting and protective immune responses to mucosal immune response. A number of studies already assessed this issue experimentally. Doses, frequency of administration, vaccine vehicles, delivery particles, as well as animal models and analytical methods of in vivo immunization vary considerably. Particle sizes as well as particle properties have been recognized as crucial parameters for bioadhesion to and adsorption from mucosal tissue (Pison et al., 2006; Vajdy and O'Hagen, 1991). The body distribution pattern of particles influencing by size of particles was a key factor to regulate the induction of systemic and mucosal immune response and the particles of diameter up to 11µm preferred to be taken up by M-cells while the fraction of taken up became zero when the particle size was at and larger than 21 µm (Tabata et al., 1996) according to mucosal cell. However, the efficacy of this transport was related to the nanosize as well regarding to nasal cells, reaching the most important transport for the smallest particle size as the intensity of the transportation was illustrated by the high nasal bioavailability of TT encapsulated into nanoparticles of 200 nm (Vila et al., 2005). Therefore, both micron and nano size achieve a prospect both for mucosal antigen delivery. Particulate surface charge has an especially important impact on mucosal adsorption as well. An optimal carrier relatively combined positive aspects but minimize problems such as biocompatibility and biodegradability. Therefore, particles made from a combination of novel type of polymer were prepared. These biodegradable polymers may be advantageous for mucosal delivery (Jung et al., 2000). The study of Thomas establishes that positive surface charges on poly(D,L-lactic-coglycolic acid) particles by stearylamine and polyethylenimine containing HBsAg enhances both the systemic and mucosal immune response upon immunization via the respiratory route of rat (Thomas, Gupta and Ahsan, 2009) which corresponded to the results from Borges that the association of the HBsAg with alginate coated chitosan nanoparticles, administered nasally to mice, gave rise to humoral mucosal immune responses, which were not induced by the HBsAg alone (Borges et al., 2008).