Research
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2. RESEARCH ENVISAGED AND PLAN OF WORK

2.1 Research Envisaged

Diphtheria is a grave upper respiratory tract disease caused by bacterium, Corynebacterium diphtheriae. The organism secretes toxin that cause inflammation of the larynx, pharynx and trachea. Further, when the toxin reaches the blood or lymphatic system, it can attack any organ in the body, and in more than 10% of cases, the disease proves to be fatal (WHO, 2009). Though diphtheria has been quite controlled using the conventional alum-adsorbed parenteral diphtheria toxoid (DTx) vaccine, yet it is ready to re-emerge again (WHO, 2009). Further, reports of diphtheria epidemics in some countries have raised serious concern of its recurrence (Galazka 2000; Kelly and Efstratiou 2003).

There has been a shift in the frequency of incidence of the disease from childhood to older age groups, in addition to the spread of disease to rural areas. This has been accompanied by several clinical symptoms, raising concerns on the epidemiological and protective efficacy of the conventional parenteral vaccine. Parenteral diphtheria vaccine lacks ability to induce long-lasting memory and require the use of multiple booster doses (Galazka 2000; Gandon et al., 2001; Clements and Griffiths 2002), thus leads to poor patient compliance. Further, it fails to induce local secretory slgA antibody response in the respiratory tract (Gandon et al., 2001). Next, the use of alum-adsorbed vaccines is associated with IgE-related hypersensitivity reactions and adverse side-effects necessitating research for other adjuvants (Martin-Munoz et al., 2002). These conditions have increased the interest in the development of a more patient-compliant, needle-free and mucosally active diphtheria vaccine, which would be apt for mass vaccination campaigns in the developing countries (Alpar et al., 2001; van der Lubben et al., 2003). Achieving this objective, though, has been constrained because of the fact that protein antigens generally induce systemic non-responsiveness rather than active immunity by mucosal administration of vaccine antigens (Mowat 2003).
Diphtheria toxoid is the formaldehyde-inactivated toxin of Corynebacterium diphtheriae, used as an active immunizing agent against diphtheria, generally in combination with tetanus toxoid and pertussis vaccine (DTP or DTaP) or with tetanus toxoid alone (Uchida et al., 1973; Collier 2001).

Oral immunization holds well-documented advantages over parenteral immunization including ease of administration, better patient compliance, ability of frequent administration and potential to induce mucosal antibody response. However, the oral delivery of antigens remains an arduous challenge because of their poor gastrointestinal absorption and susceptibility to enzymatic degradation. Consequently, the antigen is required to be administered more frequently in larger doses, thus leading to oral tolerance (Shalaby 1995; Mowat 2003; Dubois et al., 2005). Therefore, in order to induce significant immune protection, a balance between the mucosal delivery of antigens and induction of tolerance is required (Holmgren and Czerkinsky 2005).

The epithelial surfaces of both NALT and GALT have specialized antigen-sampling cells, i.e., the M cells. These M cells transport antigens from the mucosal surfaces to the underlying lymphoid tissues (Frey and Neutra 1997; Brayden et al., 2005; Misumi et al., 2009). Subsequently, the antigens are internalized and processed by antigen presenting cells (subepithelial dendritic cells and macrophages), and presented to B cells and T cells located in the mucous associated lymphoid tissues (MALT) (Brayden et al., 2005; Misumi et al., 2009). Sensitization by antigens leads to cells proliferation and switching to IgA-committed cells. These B cells ultimately leave the MALT and migrate through the systemic circulation to different mucosal sites, including the initial induction site for the terminal differentiation to slgA-producing plasma cells (Frey and Neutra 1997).

Stimulation of mucosal immune system at one mucosal site can lead to production of slgA in the local as well as at the distant mucosal sites (McGhee et al., 1992; Moyle et al., 2004). Antigen stimulation of the PPs in the
gastrointestinal tract produced slgA-producing B cells not only in the intestine, but also in the bronchi as well as in the genito-urinary tract (Nugent et al., 1998). The M-cells overlying PPs take up the gut luminal antigens by endocytosis and transport them to underlying lymphoid cells in the dome region containing functional T, B and antigen presenting cells (McGhee et al., 1992). Various cytokines, from the activated T<sub>H</sub> cells, are instrumental in activating B cells, T<sub>C</sub> cells, macrophages and several other cells that participate in the immune responses. Further, the cytokines play key role in B cells activation, proliferation, differentiation and class switching (McGhee et al., 1992; Kuby 1994). Hence, production of specific slgA and IgG antibodies, confirms the activation of T cells on oral immunization. The significant nasal and salivary slgA antibody responses are very important as the natural route of infection for diphtheria is by the respiratory mucosa. Therefore, local mucosal protection against pharyngeal carriage is likely to be decisive for preventing dissemination of disease in the populations. Conventional parenteral alum-adsorbed DTx vaccines are not able to stimulate mucosal immune responses (Shalaby 1995; Eriksson and Holmgren 2002), thus restricting their efficacy in infections of mucosal surfaces such as the respiratory tract. This also tends to clarify the rising limitations of the current vaccination schedule against diphtheria (Martin-Munoz et al., 2002). Development of oral antigen delivery systems for mucosal vaccines is a significant challenge for scientists. The instability and poor absorption of vaccine antigens in the gastrointestinal tract are the major obstacles. The main problems with oral antigen delivery like degradation in the gastrointestinal tract, poor permeability across the gastrointestinal mucosa and the first-pass metabolism greatly limits the uptake of antigens by M-cell which is a vital step for immune response (Jepson et al., 2004; George and Abraham 2006). To overcome the aforementioned obstacles, several strategies, including liposomes (Wang 1996; Okada et al., 1997; Anderson et al., 2001), micro/nanoparticles (van der Lubben et al., 2002; Vila et al., 2002; van der Lubben et al., 2003) and micro/nanoemulsion (Bielinska et al., 2007) have been
explored to encapsulate antigens for the mucosal vaccines. Among these strategies, vesicular systems and particles made of biodegradable natural polymer have gained significant interest in the past decades.

Bilosomes are non-ionic surfactant vesicles (NISVs) additionally incorporating bile salts, which function as adjuvants capable of stimulating immune responses (humoral and cellular immune response). Bilosomes are effective adjuvants comparable with Freund’s complete adjuvant, but with an improved side-effect profile, and superior in performance to alum. The bile salts function to stabilize and protect the bilosomes and its contents from the hostile environment of the gut, enabling antigen delivery via oral route (Conacher et al., 2001; Mann et al., 2006; Shukla et al., 2008).

Natural polymers, especially chitosan, have demonstrated that could enhance the immunogenicity of poor immune response antigens in the form of solution and micro/nanoparticles (Zaharoff et al., 2007; Amidi et al., 2010). Chitosan has gained increasing attention in pharmaceutical field due to several of its favorable biological properties, such as, biodegradability, non-toxicity and mucoadhesiveness (Gan et al., 2005; George and Abraham 2006). In spite of all its superior properties, chitosan has the pKa value of 5.6 and is soluble only in acidic solutions. On incubation in physiological fluid environment, chitosan loses its capacity of mucoadhesive properties and permeation enhancing effect due to the deprotonation of chitosan. Meanwhile, chitosan has limited ability for controlling the release of encapsulated macromolecule compounds because of its hydrophilic nature and solubility in acidic medium (Kotze et al., 1999; George and Abraham 2006). It might be an interesting method to overcome these obstacles by coating acid-resistant polymer, for example sodium alginate, onto the surface of chitosan microparticles. As an anionic polysaccharide, alginate can easily interact with cationic chitosan microparticles to form the polyelectrolyte complex via electrostatic interactions (Lee and Min 1996; Okada et al., 1997; Severian and Esteban 1998; Kim et al., 2002). Moreover, this coating procedure
was performed at relatively mild condition without using any organic solvent. This comparatively mild process has enabled proteins as well as cells and DNA to be incorporated into the chitosan/alginate matrices with retention of biological activity (Gombotz and Wee 1998).

The current studies aim to formulate diphtheria toxoid-loaded nanobilosomes (DTxNB) and diphtheria toxoid-loaded alginate-coated chitosan microparticles (DTxACM), and investigate the possibility of DTxNB and DTxACM as an effective vaccine formulation which could induce systemic and mucosal antibody response in conjunction with cell-mediated immune response against diphtheria. Further, the work will also endeavor to determine the oral dose of DTx using nano-bilosomes and alginate-coated chitosan microparticles that could produce serum anti-DTx antibody titres via oral route which were comparable to those following alum-adsorbed DTx intramuscular dose. The DTx containing nano-bilosomes and alginate-coated chitosan microparticles with different entrapped doses will be prepared and assessed for their potential to induce comprehensive immune protection, i.e., mucosal, systemic and cell-mediated immune responses.

2.2 Plan of Work

It was planned to identify of antigen (DTx) and quantitatively estimate it using an appropriate method. Selection of vaccine delivery systems would entail the studies on varied vesicular and microparticulate systems to attain the desired oral mucosal delivery for the selected antigen. Next, appropriate formulation components will be chosen so as to achieve the optimum formulation design with desired quality attributes. In the selection of formulation techniques(s), it was planned to screen various preparatory techniques for formulating antigen-loaded delivery systems with an aim to obtain maximum process efficiency, ease and reproducibility.

Systemic optimization studies employing FbD approaches were planned to be conducted to develop the formulation(s) under the given set of conditions to save time, effort and developmental cost, to obtain “the best” formulation(s).
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An appropriate experimental design like FCCD, Box-Behnken Design, etc. would be employed to ascertain the effect of various formulation factors on the response variables like antigen entrapment, stability in simulated gastric fluid, stability in simulated intestinal fluid, stability in bile salt solutions, etc. Response surface analysis and generation of mathematical model would be carried out using software like Design-Expert®. Quadratic and cubic polynomials would be generated using MLRA. Numerical and graphical methods like grid search, desirability function and overlay plot would be employed to search for the optimum formulation(s). The generated mathematical model would be validated by formulating the check-points (i.e., confirmatory runs), and the results would be critically compared with those predicted using FbD. Linear correlations between predicted and observed values would be explored, and statistical significance discerned.

The optimized formulation would be characterized for percent antigen entrapment, percent antigen payload, shape, size and zeta potential. Specifically, in the case of microparticles, the optimized formulations would also be characterized for surface morphology, loading efficiency and loading capacity. Furthermore, the optimized formulation would be standardized for various quality control parameters, followed by in-process and storage stability testing too. Finally, the optimized formulation are planned to be evaluated for their comprehensive immune protection efficacy by estimation of antibodies (anti-DTx IgG in serum and anti-DTx slgA in secretions) and cytokines (IL-2 and INF-γ), following oral administration of optimized formulation in varied dosage, in experimental animals.