CHAPTER 1
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One of the greatest achievements of the 20th century is prevention of numerous, potentially fatal, infectious diseases through the administration of vaccines. Vaccination against smallpox, polio, diphtheria, pertusis, tetanus, measles and other pathogens has reduced mortality more than any other disease intervention ***(Plotkin, S.L, 1994)***. Despite these successes, vaccine development has significant hurdles, both social and scientific, largely because of the nature of the prophylactic vaccine. An ideal vaccine must be 'completely' safe, easy to administer, should result in high compliance, cause little pain upon delivery, and be effective against the pathogens of the region. ***(Powel, M.F., 1996)***

To meet these requirements, research on novel vaccine development continues unabated. Mucosal strategies have emerged as a viable and attractive alternative to parenteral immunization. Advantages associated with mucosal vaccination are numerous and include the reduced cost of vaccination, patient's acceptance, reduction of the hepatic first pass metabolism and the ability to induce mucosal as well as systemic immunity. Furthermore, the immune response generated at one mucosal site is able to induce a strong immune response at most distal mucosal surfaces due to common mucosal system. ***(Westerink, M.A.J., 2002; Mestecky, J., 1987)***.

Tetanus and Diphtheria are acute, often fatal, bacterial diseases. **Tetanus** is caused by an exotoxin produced by Clostridium tetani. Two products liberated by C.tetani are the classical neurotoxin (tetanospasmin) and a haemolysin (tetanolysin) ***(Burrows, W., 1959; Weinstein, L., 1973; Hatheway, C. L., 1998)***. Tetanospasmin, a neurotoxin and the cause of the manifestations of tetanus, is a highly toxic protein that accumulates intracellularly during the logarithmic phase of growth and is released into the medium on autolysis. The toxin has an approximate molecular weight of 150,000 and is synthesized as a single polypeptide prototoxin chain. Tetanus toxin is one of the most potent known poisons on a weight basis. As little as 1ng/kg may kill a mouse, and 0.3 ng/kg will kill a guinea pig ***(Gill, D.M., 1982)***. The estimated minimum human lethal dose is less than 2.5 ng/kg. Infection usually begins with the inoculation of spores through the epithelium. Wounds accompanied by tissue injury and necrosis leading to anaerobic or hypoaerobic conditions are generally necessary for the spores to germinate and bacilli to replicate. The umbilical stump serves as a nontraumatic site where spore contamination can easily

lead to germination and bacterial replication, but traditional surgeries or piercings also can be associated with neonatal tetanus *(Bennett, J., 1999).*

Tetanus is characterized by generalized rigidity and convulsive spasms of skeletal muscles. The muscle stiffness usually involves the jaw (lockjaw), neck and then becomes generalized. The incidence of tetanus is higher in poor countries with warm and humid environment, particularly in countries near the equator and where manured soil is likely to contaminate the wound *(Abrutyn E., 1998).* Tetanus toxoid is produced by toxoidation od Tetanus toxin using formaldehyde. The prophylactic effect of tetanus vaccination using tetanus toxoid has been very marked. Before the immunization era, tetanus was observed in 2-23 per 1000 severely wounded in Europe and about twice as frequently in tropical areas. After introduction of vaccination during World War II, occurrence of tetanus decreased to 4.4 per million wounded, and during the Vietnam War tetanus was not observed in the US army *(Furst, W., 1972).* Tetanus, however, is still a major health problem in developing countries and continues to occur even in countries with high medical standard *(WHO, 1986).* In countries where primary vaccination has been carried out for years, tetanus is mainly observed among the elderly and non-immunized. *(Simonsen, O., 1987).* Tetanus kills on an average 140 times more individuals in poor developing countries than in rich developed countries. Incidence of tetanus is higher by a factor of 5-8 times in rural than in urban settings. About 30% of all the cases of tetanus and 80% of deaths from tetanus are recorded in newborn children in the developing countries because of the lack of good hygienic practices. Another group, which is more prone to tetanus is of people above 60 years of age.

Currently, in the world, neonatal deaths reported due to tetanus are about 2,00,000 per year, in spite of the fact that an economical, effective and safe prophylactic agent has been available for more than 50 years! There are about 57 countries where tetanus is considered as one of the major diseases. India reports maximum number of cases in the world every year, and is leading the list of class 'C' countries which are declared as countries wherein tetanus is still a major issue as per UNICEF *(www.unicef.org; Wassilak S. G. F, 2002).*

Diphtheria is a rapidly developing, acute, bacterial disease, involving both local and systemic pathology, which is caused by *Corynebacterium diphtheriae,* a gram-positive bacillus. Routine immunization against diphtheria, introduced in the 1940s, led to the almost complete eradication of this disease from developed countries by the 1970s.

However, a high proportion of the European population has been shown to have diphtheria antitoxin titres below the putative protective level *(Rappuoli R., 1988)*. As a result, there has been a resurgence of diphtheria in several European countries in the 1980s and 1990s *(Rappuoli R., 1988, Galazaka, A.M., 1996)*.

Diphtheria is endemic in many developing countries. Almost 120 countries report cases with diphtheria to WHO every year. The disease is seen mainly in children between the age of 2-5 years. Dismal performance on immunization front is reflected in continuing high incidence of the disease in India. The annual reported incidence of diphtheria in India has varied from 15,000 to 35,000 cases with an average of 25,000 per year. It is a disease of rural settings and that of schools and other institutions where children of susceptible age group are herded together. Recently, a massive epidemic of diphtheria occurred in the former Soviet Union, causing more than 50,000 cases and several thousands deaths during the last 4 years. This showed that vaccination of infants and boosters for adults with effective vaccines is still absolutely necessary to keep diphtheria under control. *(Wharton, M., 2002; www.who.int)*

The illness is characterized by a membranous inflammation of the upper respiratory tract, usually of the pharynx but sometimes of the posterior nasal passages, larynx, and trachea, and by widespread damage to other organs, primarily the myocardium and peripheral nerves. Most of the clinical symptoms of the disease are due to the release of the potent diphtheria toxin from the lysogenized strains of the bacteria. Extensive membranes and organ damage are caused by local and systemic action of a potent exotoxin. Prevention may, therefore, be obtained by toxin neutralizing antibodies (antitoxin), induced through active immunization with non-toxic forms of the toxin. Current diphtheria vaccines are prepared by converting diphtheria toxin to its non-toxic, but antigenic, toxoid by formaldehyde treatment and are mostly combined with tetanus toxoid and whole-cell or acellular pertusis vaccines for infant immunization *(Rappuoli R., 1998)*.

In most countries it is now recommended that booster doses of diphtheria vaccine be administered every 10 years. However, a limiting factor to public acceptance could be adverse effects associated with the vaccine, due to the presence of accessory antigens in crude or partially purified toxoid preparations like development of local reactions, mild to moderate pain at the injection site and fever *(Relyveld, E., 1997)*. Both diseases, Tetanus and Diphtheria, can be prevented solely by the presence of toxin-neutralizing

antibodies, which can be induced through active immunization with nontoxic forms of the toxins or provided by passive immunization.

Vaccines against tetanus and diphtheria require more than one dose and booster dose for long term protective immunity. The multiple dose parenteral administration schedules of these vaccines increases the cost of immunization due to need for maintenance of cold chain, requirement of skilled person, inconvenience and incomplete subject compliance. Diphtheria (D) and tetanus (T) vaccines are presently still prepared using the formaldehyde toxoidation method and used mostly combined with whole-cell pertusis (P) for infant immunization (DTP). TD vaccine is used in some countries that have abandoned the use of the reactogenic pertusis vaccine, while T and Td (tetanus containing a low dose diphtheria) are used to boost immunity in adults. The above vaccines have a low purity and are associated with some undesired side effects when administered via parenteral route. *(www.immunize.org; Wharton, M., 2002; Rappuoli, R., 1998; www.worldwidevaccines.com;)*

The most desirable route of administration is the oral route. The major advantages of oral route are ease of administration, safety, less dependency on skilled medical person and no need of sterile conditions during administration. Morever, with respect to use of vaccines, mucosal delivery of vaccines minimizes adverse effects and allows for easier administration, making vaccination in the home a possibility *(O'Hagan D.T., 1998; Walker, R.I., 1994).* Mucosal immunization would therefore be of particular benefit where frequent boosting is required, as is the case for diphtheria and tetanus. Mucosal immunization has the advantage over conventional parenteral immunization of stimulating both systemic and mucosal immunity. However, soluble antigens administered mucosally tend to elicit poor immune responses and require the use of delivery vehicles like microparticles, immunostimulants or adjuvants to increase immunogenicity. *(McNeela, E.A., 2001)*

The normal route by which antigen is taken up by the gut associated lymphoid tissue (GALT) is via the epithelial surface. The predominant site for antigen uptake in immunogenic form is through the modified epithelium overlying the Peyer's patches *(Owen, R.L., 1977)* transported through M cells to underlying dendritic and lymphoid cells. **Peyer's Patches (PPs)** are the main target for oral vaccines, which are present in the lower ileum. The intestinal epithelium overlying the PPs is specialized to allow the transport of pathogens into the lymphoid tissue.

PPs are collections of lymphoid follicles, which are separated from the intestinal lumen by a single layer of specialized epithelium containing M cells and enterocytes. This epithelium is different from the villus epithelium in that the enterocytes are more cuboidal, it contains fewer goblet cells, there is no secretory component *(Abe, K., 1977)*, and it has reduced activity for some hydrolases in the apical membrane *(Smith, M.W., 1985)*. PPs play a central role in antigen uptake and induction of an immune response. Generally, PPs are located at the antimesentric border of the small intestine *(Karali, T.T., 1995)*. In general, the ileum contains larger and more numerous patches than the jejunum, where as the duodenum contains very few patches. M cells of the human PPs dome epithelium comprise less than 10% of the total dome epithelial cells. *(Yeh, P.Y., 1998)* M cells use multiple endocytic mechanisms for uptake of macromolecules, particulates and microorganisms. *(Yeh, P.Y., 1998)*

Following stimulation by an antigen in PPs and its presentation to B- and T-cells, they proliferate and subsequently leave the PPs via efferent lymphatics and reach the systemic circulation through the thoracic duct. Empirical experiences with mucosal immunization has resulted in a generally accepted conclusion that considerably higher doses of antigens are required. This is due to the elimination of antigens, existence of effective mechanical (epithelial cells) and chemical (mucins) barriers, degradation and denaturation of antigens by enzymes and acids. Thus, only minute quantities of fully potent antigens reach the mucosal lymphoid tissues.

Several strategies are proposed to circumvent these problems e.g., Muramyl dipeptides and related adjuvant molecules; Cholera toxin and cholera toxin B subunit; colonization of PP's with genetically engineered strains of Salmonella, E. coli, Lactobacilli, BCG; Liposomes; Microencapsulation. *(Mestecky, J., 1994; McGhee, J.R.,1990; Mestecky, J.,1987; McGhee, J.R.,1992; Mestecky, J.,1991)* The advantages of microencapsulation are : Immune response may be enhanced by increased uptake by PP's (cationization of microcapsule surfaces); incorporated antigens are protected from digestion; both mucosal and systemic immune response may be induced depending on the size of the microcapsules; programmed release (combination of fast and slow) of antigens may induce both primary as well as booster responses by single immunization; biocompatible materials are used in microcapsules; eliminates problems with vaccine storage and delivery (no needles; syringes, or health personnel necessary)

However, disadvantages include degradation of sensitive antigens by organic solvents during the preparation of microcapsules; expense in preparation; limited uptake by gastrointestinal associated lymphatic system.

Several types of microparticles have been demonstrated to significantly enhance the systemic and /or mucosal immune system. Association of the vaccine with microparticulate drug carrier system may prevent its degradation in the stomach and the gut and may stimulate the M-cells to transport the vaccine to the dome of the PPs, where the microparticles are degraded and the vaccine is released into lymphoid tissue. The uptake of nano-and microparticles by M cells in Peyer's patches has been well documented. The uptake efficiency of PPs is mainly dependent on the size of the microparticles. Particles lesser than of 10μ size are taken up by the PPs and particles larger than 10μ are lodged on to the PPs. ***(Jani, P., 1989; Pappo, J., 1989; Kreuter, J., 1991; Scherer, D., 1993, O'Hagan, D.T., 1989).***

Chitosan is a biodegradable, soft tissue compatible, mucoadhesive polysaccharide ***(Aspden, T. J., 1997).*** It has been widely used in pharmaceutical research and in industry as a carrier for drug delivery and as biomedical material ***(Mao, H.Q., 2001).*** As a drug carrier, chitosan has been formulated into different pharmaceutical dosage forms such as tablets ***(Upadrashta, S.M., 1998),*** beads ***(Chandy, T., 1992),*** microspheres ***(van der Lubben, I.M., 2003)*** and nanoparticles ***(Fernandez, U.R.,1999).*** An advantage of chitosan microparticles is that the loading is performed by incorporation in an aqueous solution. Therefore, the antigen is not exposed to organic solvents. ***(van der Lubben, I.M., 2003)*** Recently, it was shown that chitosan microparticles and nanoparticles are able to entrap large quantities of antigens. ***(van der Lubben IM, 2002; Fini, A., 2003; Singla, A.K.,2001).***

The Aim of the present project was to prepare chitosan based microparticle oral delivery system of Tetanus Toxoid (TT) and Diphtheria Toxoid (DT) for the purpose of targeting the PPs based on the following **hypothesis:**

1. Encapsulation of toxoids in chitosan microparticles will protect toxoids from degradation in GIT.
2. The microparticles will be taken up by PPs and increase uptake of the vaccines.
3. Subsequent booster administration will lead to the formation of memory cells.
4. Mucoadhesivity of chitosan will increase the residence and contact time of the system leading to better absorption of TT and DT.

The main focus on which the project was based were the several advantages of chitosan like biodegradable, mucoadhesive and very high loading capacity. Its also well documented that as Chitosan is a polycationic polymer, its reaction with negatively charged components, either ions or molecules, can lead to the formation of a crosslinked network through ionic bridges between polymeric chains. As ionic crosslinking is a simple and mild procedure, it can be considered safe for entrapping bacterial vaccines, **Tetanus Toxoid** and **Diphtheria Toxoid.**

Plan of work:

1. Preformulation studies for the selection of compatible carriers and additives.
2. Development of suitable sensitive, accurate and reproducible analytical method for the estimation of toxoids.
3. Optimization of parameter for the formation of chitosan microparticles.
4. Study of the effect of variables on the entrapment efficiency of TT and DT.
5. Optimization of TT and DT entrapment to obtain stable and reproducible formulation with desirable particle size
6. In vitro characterization of the loaded microparticles.
7. Stability studies of formulation in gastric and intestinal conditions and at room temperature.
8. Development of methods for quantitative estimation of TT/DT specific antibodies like IgG from serum and IgA from intestinal lavage, intestinal washings and fecal matter extracts.
9. In vivo performance evaluation studies of orally administered optimized TT and DT formulations.

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- Unicef web: www.childinfo.org
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