# CHAPTER 2
# LITERATURE REVIEW

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## 2.1 SYSTEMIC AND MUCOSAL IMMUNITY

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).*

### 2.1.1. Immunity:

The term 'immunity' refers to the resistance exhibited by the host towards injury caused by microorganisms and their products. Immunity against infectious diseases is of different types:

1. Innate Immunity:
    a. Nonspecific (Species, Racial, Individual)
    b. Specific (Species, Racial, Individual)
2. Acquired Immunity
    a. Active (natural, artificial)
    b. Passive (natural, artificial)

Several factors influence the level of innate immunity in an individual: Age, Hormonal Influences, Nutrition. The type of immunity most commonly encountered is naturally acquired active immunity to pathogens. *(Ananthnarayan, R., 1997)* However, diverse pathological effects have also been shown to arise from immune responses to nontoxic and noninfectious antigens i.e. macromolecules that will induce the formation of the formation of immunoglobulins or sensitized cells that react specifically with the antigens. Thus the immune response is directly responsible for allergies, autoimmune diseases (e.g., rheumatoid arthritis), and graft rejection. Also, the failure of various aspects of the immune system can result in the development of malignancies and death due to overwhelming infections. *(Pelczar, M.J., 1993)*

The immune system is comprised of a single integrated cellular system producing effector products of two types: serum antibodies that constitute part of the humoral immunity and sensitized cells called lymphocytes that constitute cell-mediated immunity. While antibodies are effective in opsonizing bacteria and neutralizing toxins and viruses, lymphocytes are important in eliminating intracellular parasites and viruses

and rejecting tumors and transplants. Thus the immune response, in eliciting reactive antibodies and cells in response to antigens, not only forms the principal means of defense in vertebrates against infection by pathogenic microorganisms and larger parasites, but also acts as a surveillance mechanism against the transformation of host cells into cancer cells. (*Stanier, R.Y., 1995*)

As mentioned above, immune responses are processes in which animals form specifically reactive proteins (antibodies) and cells in response to great variety of foreign organic macromolecules and molecules. The generalized immune response has four primary characteristics: discrimination, specificity, anamnesis, and transferability by living cells. The first primary characteristic refers to the ability of the immune system to discriminate between "self" and "non self", and therefore it responds only to materials, which are foreign to the host. Second, the response is highly specific for the inducing material or antigen to which the immune antibodies or cells will react in greatest strength. The third characteristic refers to the ability to elicit a larger specific response more quickly when induced by a second exposure to the same foreign antigen. This is called immunologic memory or the anamnestic response. Finally, active immunity is only transferable from one inbred animal to another by the immune cells or lymphocytes and not by serum. Whereas immune response is capable of temporarily transferring passive immunity, transfer of active immunity requires the long-term regenerative ability of living cells. (*Pelczar, M.J., 1993; Stanier, R.Y., 1995*)

Some nonspecific materials, such as mineral oil and alum, have the ability to prolong and intensify the immune response to a specific antigen when they are injected together with the antigen. Such materials are called adjuvants because they help the immune response.

**2.1.2. Antigen:**

An antigen is any substance which, when induced into the vertebrate host, stimulates the production of antibodies and reacts with preformed antibodies, if they are already present. This demonstrates the two properties of antigens: Specificity and immunogenicity. The term antigenicity and immunogenicity differ in that the former refers to the "foreignness" of a substance or its specific reactivity while the latter refers to the ability of a substance to stimulate an immune response.

Antigens are generally proteins or polysaccharides with molecular weights greater than 10,000 daltons. They may be nucleoproteins, lipoproteins, glycoproteins from any biological source, or synthetic polypeptides or polysaccharides.

The antigens which are primarily concerned are those of microbial origin. The exotoxins produced by *Corynebacterium diphtheriae, Clostridium tetani,* and certain other microorganisms are potent antigens that stimulate the production of antitoxins (toxin specific antibodies) in the body of the host. Most pathogenic bacteria, viruses, and rickettsias are good antigens, which stimulate the production of antibodies capable of protecting against infection and which are useful in diagnostic tests. Heat or chemical treatment destroys the viability of most microorganisms without necessarily decreasing or altering their antigenicity.

**2.1.3. Vaccine:**

Vaccine, which are suspensions of killed, living, or attenuated (having weakened virulence) cultures of microorganisms, are used as antigens to produce immunity against infection due to the particular microorganism. Typhoid fever vaccine consist of killed cells of *Salmonella typhi.* Toxoids are made form extracellular toxind (exotoxins) by destroying the poisonous portion with heat, ultraviolet light or chemicals without altering their antigenic specificity and often enhancing their immunogenicity. Toxoids made from toxin-producing microorganisms such as *C. tetani* and *C. diphtheriae* are used to immunize against the harmful effects of diphtheria and tetanus, respectively.( ***Pelczar, M.J., 1993; Ananthnarayan, R., 1997***) The events following the administration of vaccine formulation are now well understood. When it is injected into the body, it is first recognized as "foreign" by macrophages and other phagocytic cells. Upon being ingested by macrophages, its protein chain is digested into fragments, some of which are loaded on to MHC proteins. Phagocytes also carry the Ag to lymphoid tissue where B and T cells conjugate. The ligands for the receptors on these cells are recognized, and the immune response is initiated.

**2.1.4. Antibodies:**

Specific acquired immunity against infection is primarily a property of a group of serum glycoproteins (proteins with attached carbohydrate) called antibodies. These antibodies have been produced by subpopulation of white blood cells in theimmune system called lymphocytes. These are small round cells, 6 to 7 μm in diameter, with a high nuclear-to-cytoplasmic ratio in their resting stage, and they are capable of expanding greatly in

volume and activity in response to an antigen. This process is called lymphocyte activation.

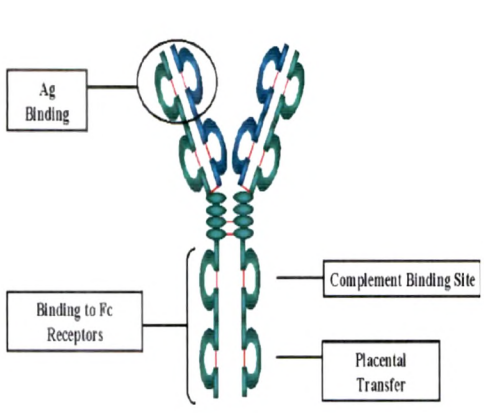
Antibodies are termed as immunoglobulins (Ig) since they are globular proteins with immune function, the bulk of which generally separate in the gamma region on electrophoresis.

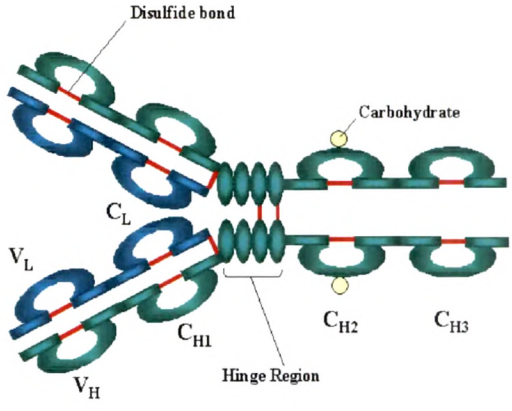
**2.1.4.1 Structure and function of antibodies:**

All antibody molecules have two basic functions: 1. antigen binding and 2. participation in effector functions depending upon the physical properties of the antibody. These properties of antibodies can be separated by cleavage of the antibody molecule with proteolytic enzyme papain. This produces two antigen-binding fragments (named Fab, or antigen binding fragment) and single crystallizable fragment (termed Fc, or crystallizable fragment),which possesses the physical properties of the molecule (**Figure 1**). Only the Fc fragment has been shown to fix complement and bind to Fc receptors, and the Fab does not interact.

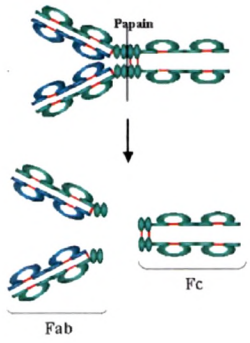
There are five separate classes of immunoglobulins: immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin D (IgD) and immunoglobulin E (IgE) (**Table 1**). Their respective heavy chains are designated by the Greek letters gamma (γ), mu (μ), alpha (α), delta (δ) and epsilon (ε). Some of these immunoglobulins are polymers of the basic unit (**Figure 1**), and some have subclasses. Each immunoglobulin is linked with one of two types of light chains called kappa (κ) or lambda (λ).

Figure 1: Immunoglobulin fragments: structure and function relationship




- Fab
  - Ag binding
  - Valence = 1
  - Specificty determined by $V_H$ and $V_L$
- Fc
  - Effector functions



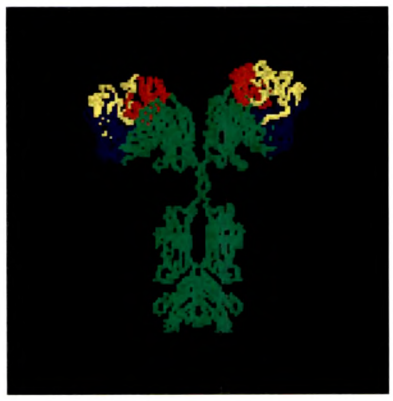



**Table1: Some properties of Immunoglobulin Classes**

*(Pelczar, M.J., 1993; Ananthnarayan, R., 1997)*

| | IgG | IgA | IgM | IgD | IgE |
|---|---|---|---|---|---|
| **Site found** | Internal Body fluids, particularly extra vascular | Serum, external body secretions | Largely confined to bloodstream | Serum, on lymphocyte surface of newborn | Serum |
| **Heavy-Chain Designation** | γ | μ | α | δ | ε |
| **Sub Classes** | 4 | 2 | 1 | 1 | 1 |
| **J-chain** | - | + (pIgA) | + | - | - |
| **Sedimentation Coefficient (s)** | 7 | 7 | 19 | 7 | 8 |
| **Molecular Weight** | 150,000 | 160,000 (Serum) (160,000)n serum 390,000 (secretions) | 900,000 | 180,000 | 190,000 |
| **Serum Concentration (mg/ml)** | 12 | 2 | 1.2 | 0.003 | 0.00004 |
| **Half life (days)** | 23 | 6 | 5 | 2-8 | 15 |
| **Daily Production (mg/kg)** | 34 | 24 | 3.3 | 0.4 | 0.0023 |
| **Intravascular distribution (%)** | 45 | 42 | 80 | 75 | 50 |
| **Carbohydrate (%)** | 3 | 8 | 12 | 13 | 12 |
| **Complement Fixation** | | | | | |
| **Classical** | ++ | - | +++ | - | - |
| **Alternative** | - | + | - | - | - |
| **Placental Transport** | + | - | - | - | - |
| **Present in Milk** | + | - | - | - | |
| **Selective secretion by seromucus glands** | - | + | - | - | - |
| **Heat stability** | + | + | + | + | - |
| **Functions** | Major line of defense against infection during the first few weeks of a baby's life; neutralizes bacterial toxins; binds to microorganism to enhance their phagocytosis and lysis | Protects mucosal surfaces from invasion by pathogenic microbes | Efficient agglutinating and cytolytic agent; effective first line of defense in cases of bacteremia | Regulator for the synthesis of other immunogl-obulins; fetal antigen receptor | Responsib-le for severe acute and occasional-ly fatal allergic reactions; combats parasitic infections |

### 2.1.5. Immunization:

Immunization is the artificial induction of immunity to disease. The remarkably successful vaccination program designed by World Health Organization has been implemented as Expanded Program of Immunization since 1974. Immunization is of two types: Passive and Active immunization. Antigenic material that produces specific immunity by stimulating an immune response is termed as Vaccine. Usually, an individual who has been never been exposed to an antigen lacks detectable amounts of the corresponding antibody. Then, following exposure by injection or some method, a primary response occurs: the level of antibody usually remains undetectable for several days, and then it rises over a period of one or more weeks, reaching a peak, and finally, gradually decreasing. When the individual is exposed again to the same antigen, a response termed a secondary or anamnestic response follows in most cases: the level of antibody begins to rise much sooner than it did before, and it reaches a much higher value. The physiological basis of the secondary response is the proliferation of specific $T_H$- cells and B-cells that occurs during the primary response. These cells have a long lifespan and are therefore able to serve as memory cells in the secondary response. It is these cells that are responsible in many cases for acquired immunity to an infectious disease. (*Stanier, R.Y., 1995; Cruickshank, R., 1968*).

### 2.1.6. Impact of Current Vaccines:

The use of vaccines against infectious diseases has been one of the true success stories of modern medicine. This is best exemplified by the fact that there has been a 90 to 100% decline in mortality and morbidity with several childhood infections since the introduction of vaccines and their universal use in children. It is remarkable that no case of smallpox has been reported in the world during the past three decades, and poliomyelitis has now been eradicated from Europe and the North American hemisphere and most other parts of the world. Similarly impressive is the observation that over the past 50 years, there has been a 95 to 100% reduction in morbidity and mortality associated with diphtheria, pertussis, tetanus, *Haemophilus influenzae* type B, measles, mumps, and rubella in the United States. Although these accomplishments have not been inexpensive from a financial standpoint, the costs of vaccines and other preventive public health measures represent a small fraction of the national per capita income, and the cost-benefit ratios of their use have ranged from 1:2 (pertussis) to 1:10 or more for poliomyelitis and measles *(CDC, 1998)*. To date, over 25 vaccines have become

available for human use. With the exception of vaccines against poliomyelitis (Sabin oral polio vaccine [OPV]), typhoid, cholera, adenovirus, and live reassortant rotavirus vaccine, all currently available vaccines are licensed for use only via a nonmucosal route, usually subcutaneous or intramuscular inoculation. Although many vaccine-preventable diseases have been controlled in the developed world, such diseases continue to pose major public health problems in the economically underprivileged countries. Furthermore, during the past two decades, a number of new human infectious diseases have been identified. Severe infections with otherwise low-virulence pathogens and some infections believed to have been extinct have reemerged with increasing frequency in many parts of the world, including the United States. With increasing use of antibiotics in veterinary and agricultural products and for prophylaxis and treatment of infectious diseases in humans, a significant increase in the emergence of antibiotic-resistant organisms, especially *Streptococcus pneumoniae*, enterococci, and gram-negative enteric pathogens, has been observed worldwide. *(CDC, 1998)*. Thus, concerns about emergence of new pathogens or reemergence of old infectious diseases reinforce the need for availability of additional vaccines to prevent existing as well as emerging infectious diseases identified to date. However, despite and to some extent because of the remarkable progress in the vaccine development and delivery systems, it is apparent that other immunization strategies need to be explored in order to simplify the increasingly complex childhood vaccination schedules. It is estimated that about 11,000 children are born each day in the United States. Each child will require 20 to 25 vaccinations by about 18 months of age. These vaccines are generally administered via nonmucosal or percutaneous injections *(CDC, 1999)*.

**2.1.7. Limitations of the current Modes of Vaccination:**

**2.1.7.1 Lack of compliance with the multi injection immunization schedule:**

Immunization with several vaccines, such as the triple vaccine diphtheria-pertussis-tetanus (DPT), widely used in the Extended Program of Immunization (EPI) demands administration of the vaccines at repeat intervals of 4-6 weeks three times. The multiple injection has several limitations. A percentage of subjects who receive the first dose do not turn up for the second or third injection, and are therefore not rendered "immune" to the disease. It is estimated that one child out of every five who receives the first dose does not complete the immunization schedule and is not fully immunized. Thus the lack of compliance to the multi injection schedule is one of the main limitation of the current

mode of vaccination. A major international program of research under WHO seeks to develop a single vaccine to be given at a single contact point to children for preventing 4-6 diseases. Oral route immunization has been explored for the immunization, which may increase the compliance with schedule due to ease and self administration.

**2.1.7.2 Complicated Diseases Require Peripheral and Mucosal Protective Immunity:**

Protections from diseases that require complex forms of immunity require consideration of multiple "ancillary" factors. Most bacterial and viral disease access to the host via mucosal tissues and these include human immunodeficiency virus (HIV) and cholera. The fact that most infectious diseases are encountered through mucosal tissues has posed new challenges in the development of vaccines. Many currently used vaccines induce systemic immune responses only, which are ineffective against mucosal infections. ***(Challacombe, S.J., 1997)***

The nature of the vaccine antigen and route of its administration may confer anatomic compartmentalization to the immune response, restricting it to peripheral or mucosal immunity. Some infectious disease organisms spread parenterally to involve parenchymal organs but do not infect the epithelial cells lining of respiratory, gastrointestinal or urogenital tracts. Protection against these pathogens depends on circulating IgM and IgG antibodies and/or peripheral cell-mediated immunity for resolution. Other disease agents principally infect mucosal epithelium, causing serious nutritional and pathophysiological alterations; but, in these diseases, circulating antibodies of the IgG class are not protective. Instead, secretory IgA and/or intraepithelial cytotoxic lymphocytes are needed for protection.

Once a multitropic pathogen enters the body across the mucosae of the eyes, nose, throat, lungs and gastrointestinal tract, it might be able to disseminate and initiate systemic parenchymal as well as mucosal pathology. Parenteral immunization, the most common route of vaccination, usually elicits a peripheral immune response, with protective IgM/IgG antibodies and peripheral cell-mediated immunity. Parenteral immunization usually fails to stimulate mucosal lymphatic tissues to generate protective IgA antibodies or antigen-specific IEL. Many hazardous agents infect or intoxicate across the mucosae but spread through the systemic circulation. Protection against these agents requires vaccines that induce both peripheral and mucosal immune responses. There is a significant contribution from local T cells in the induction of locally produced IgA

antibodies rather than plasma derived antibodies and in most case their induction requires direct mucosal immunization.

**2.1.7.3 Side effects:**

Parenteral administration is usually associated with the mild to sever side effects. In case of Tetanus vaccination mild local reactions are relatively common after receipt of tetanus toxoid, more serious reactions, including neurologic and hypersensitivity reactions, are also observed. The rates and severity of adverse events in recipients of tetanus toxoid are influenced by the number of prior doses and level of pre-existing antitoxin, perhaps the amount of toxoid in the dose, the type and quantity of adjuvant, the route of injection, the presence of other antigens in the preparation, and perhaps the presence of organo mercurials used as preservative. The most common adverse event following injection of tetanus toxoid is a local reaction (0% to 95% of recipients). ***(Myers, M.G., 1982)*** Among recipients of a booster dose of adsorbed toxoid, 50% to 85% experience pain or tenderness at the injection site and 25% to 30% experience edema and erythema. More severe local reactions characterized by marked swelling occur in fewer than 2 % of vaccinees. ***(McComb, J.A., 1961; Eisen, A.H., 1963; Levine, L., 1981)***

Aluminum adjuvant in adsorbed products theoretically can invoke local inflammatory responses more frequently than fluid toxoid because the presence of adjuvant can induce activation of complement and stimulate macrophages. ***(Collier, I.H., 1979; Edelman, R., 1980)***. Aluminum adjuvant can cause sterile abscesses when given subcutaneously. ***(Edelman, R., 1980)*** Use of jet injectors, which deposit some toxoid in the subcutaneous tissue, results in twofold higher rates of edema at the administration site than intramuscular injection by needle.

Fever can accompany a local response. Other systemic symptoms such as headache or malaise are reported less frequently than fever. Lymphadenopathy also can occur after toxoid inoculation. ***(Eisen, A.H., 1963)*** Peripheral neuropathy, particularly brachial plexus neuropathy, has been reported to occur hours to weeks after tetanus toxoid administration ***(Quast, U., 1979)***. Tetanus toxoid occasionally induces an immunoglobulin E (IgE) response, particularly with aluminum salt-adsorbed toxoid ***(Vassilev, T.L., 1978; Nagel, J., 1977; Matuhasi, T., 1982)*** True anaphylactic (type I hypersensitivity) reactions to purified tetanus toxoid are rare ***(Inst Med Vac Safety Comm, 1994)***.

**2.1.7.4 Cross Regulation:**

Route of vaccination is crucial for determining whether protective peripheral or mucosal immunity would develop. Furthermore, immunization methods and routes for inducing mucosal immunity often delayed or prevented induction of peripheral immunity and vice versa *(MacDonald, T.T., 1982)*. This phenomenon, "cross regulation (**Figure 2**), was first revealed in its bimodal form by Pierce and Koster *(Pierce, N.F., 1978)* and *Hamilton, S.R., (1979)* when they were testing a non-toxic cholera vaccine candidate. They found that initial priming by vaccinating parenterally reduced the ability of the vaccine to elicit immunity in the mucosae when the second inoculation of antigen was given by an enteric route. But parenteral priming resulted in a strong booster response when the second dose of antigen was also given parenterally.

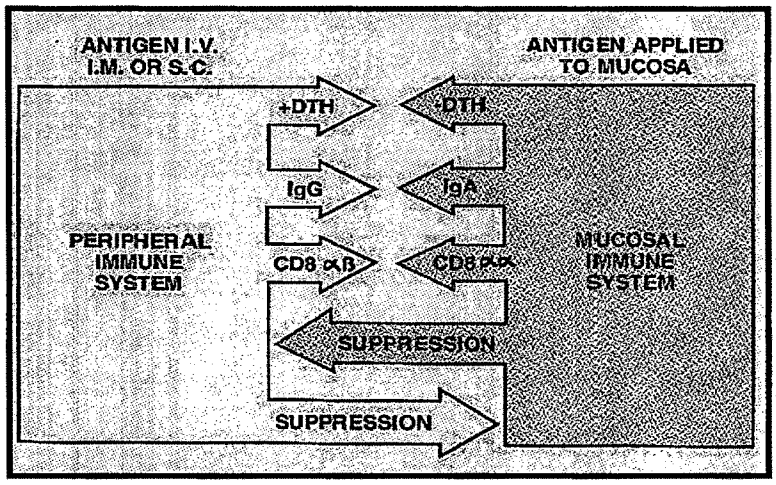



**Figure 2 : Cross regulation between Peripheral and Mucosal Systems**

**2.1.7.5 Process, Storage and administration to patients:** Parenteral vaccines are produced under aseptic conditions and need to be administered using sterile needles under the supervision of competent medical personnel, which increases the cost of vaccination. Vaccines are generally stored at 4-8$^{0}$C, which requires cold chain distribution and storage. This may be a limiting factor in developing and third world countries, where it may not be possible, thus reducing the immunization coverage.

### 2.1.8. Mucosal Immunity:

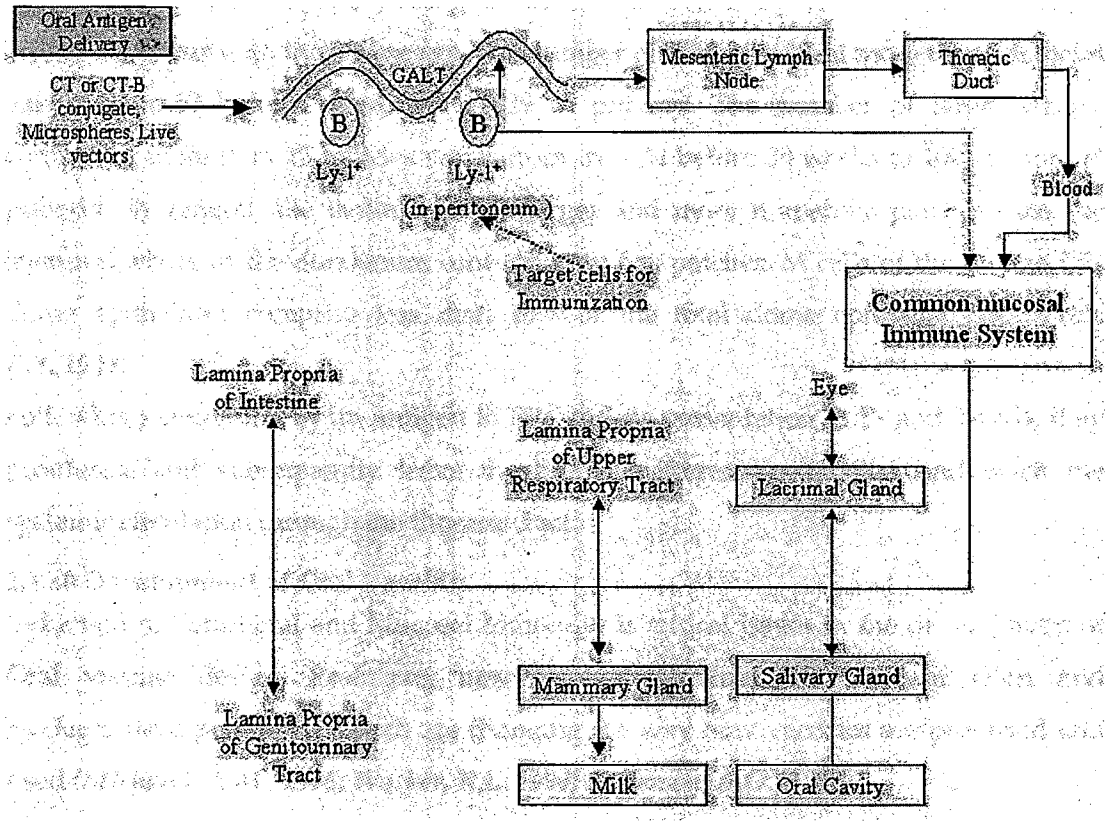
Due to above mentioned limitations of parenteral route of administration of conventional vaccines, mucosal route of administration was explored by many researchers for successful administration of antigens. Immunoprophylaxis by the mucosal route is an important approach to controlling mucosally acquired infections. (Ogra, P. L., 2001). The ability to induce a balanced systemic and secretory immune response following immunization is determined by complex set of interacting factors. These include nature of the antigens and route of administration, the nature of the mucosal microenvironment, the immunologic vehicle employed for vaccine delivery, and effects of bystander immunologic and antigen-related events occurring concurrently in the mucosal environment.

The mucosal vaccines currently approved for human use include typhoid, cholera, adenovirus, OPV, and rotavirus.

Oral vaccine delivery offers significant advantages over conventional parenteral routes. Most notably, oral delivery offers the potential for stimulating mucosal responses that protect against pathogen invasion at mucosal surfaces. In addition, oral vaccines are easy to administer, not requiring sterile needles not trained personnel, convenience and cost factors of considerable significance in developing countries. **(Clark, A.M., 2002)**

The mucosal surfaces of the gastrointestinal and respiratory tracts represent the principal portals of entry for most human pathogens. Direct inoculation of pathogens into the bloodstream and sexual contact are other important routes of infection. Most external mucosal surfaces are replete with organized follicles and scattered antigen-reactive or sensitized lymphoid elements, including B cells, T lymphocytes, T-cell subsets, plasma cells, and a variety of other cellular elements involved in the induction and maintenance of immune response. The mucosal surfaces represent a critical component of the mammalian immunologic repertoire. The major antibody isotype in external secretions is secretory immunoglobulin A (sIgA) **Figure 3.** Approximately 40 mg of IgA per kg of body weight is secreted daily, especially from the gastrointestinal tract, and the total amount of IgA synthesized is almost twice the amount of IgG produced daily in humans. It is, however, interesting that the major effector cells in the mucosal surfaces are not IgA B cells, but T lymphocytes of CD41 as well as CD81 phenotypes. It is estimated that T lymphocytes may represent up to 80% of the entire mucosal lymphoid cell population ***(Conley, M. E., 1987)***.

**Figure 4: Common Mucosal system**




The normal route by which antigen is taken up by the gut associated lymphoid tissue 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.,1974)*** 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, i.e. FAE. 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. The number and location of PPs varies between species, but the basic morphological structure is very similar. Generally, PPs are located at the antimesentric border of the small intestine ***(Karali, T.T.,1995)***.

vaccine stimulates the appropriate system or a combination of systems, immunity might not be complete. The concept of anatomic compartmentalization of immunity is supported by observations from several disciplines *(Kroemer, G., 1993)*.

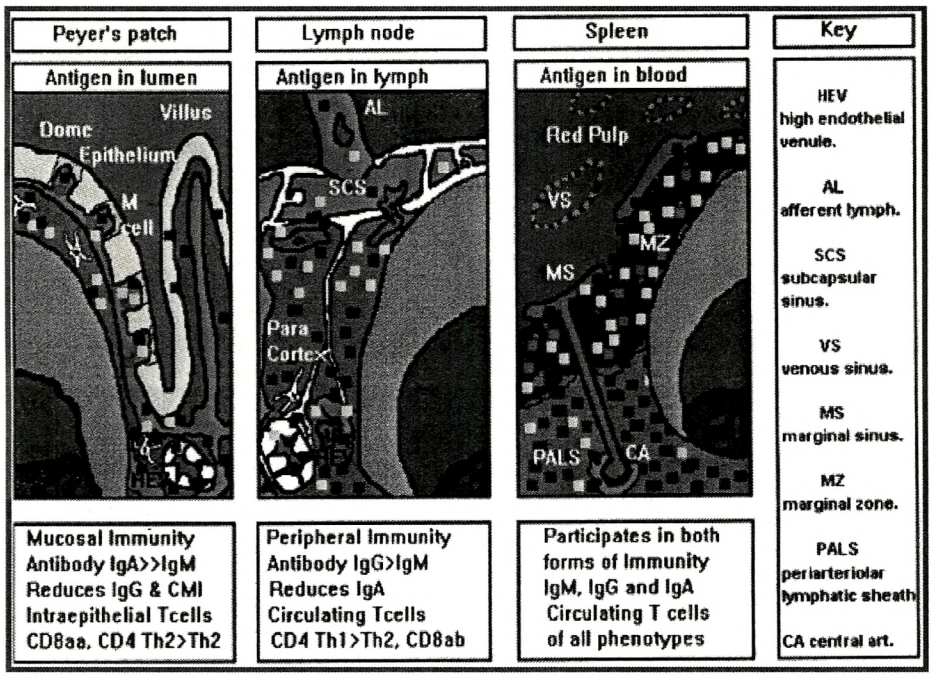
The way antigens are acquired by individual lymphatic tissues affects the outcome of an immune response. For example, the same antigen may produce qualitatively different immune responses in lymph nodes, spleen or Peyer's patches as shown in **Figure 5** *(Anderson, A.O., 1990)*. Antigens in lymph are filtered, trapped, processed and presented where the lymph passes over fixed antigen-presenting cells in lymph nodes. Such antigen handling by lymph nodes most often results in "peripheral immunity," characterized by the appearance of specific IgG in the blood. Antigens in blood are filtered, trapped, processed and presented in strategic blood/tissue interfaces in the spleen. This also results in "peripheral immunity". However, the spleen microenvironment is somewhat more complicated because it also accommodates circulating antigen-presenting cells and immunoreactive T- and B cells from other tissues committed to either peripheral or mucosal immunity. Antigens in the lumens of enteric organs (i.e., the respiratory and gastrointestinal tracts) are non-destructively endocytosed by specialized epithelial cells called "M" cells (**Figure 6**) and transcytosed onto lymphoid cells in Peyer's patches where response to antigen presentation triggers commitment to "mucosal immunity" characterized by release of specific IgA into the secretions. M cells use multiple endocytic mechanisms for uptake of macromolecules, particulates and microorganisms. They can carry out fluid phase endocytosis, adsorptive endocytosis and phagocytosis. Each of these processes results in transport of material into endosomes and large multivesicular bodies, followed by exocytosis across the basolateral membrane. Transcytotic vesicles only have to travel few microns from the apical to the basolateral surface and the whole process of the trnascytosis can take as little as 30-60 min. *(Yeh, P.Y.,1998)*

Lymphocyte traffic patterns, regulated by selective expression of adhesion proteins in peripheral or mucosal lymphatic tissues, maintain anatomic segregation of immunological memory by causing antigen-primed cells to return to specific anatomic.

destinations where they will encounter conditions that further facilitate expression of peripheral or mucosal immunity *(Kantele, A., 1997)*. Among potentially myriad factors, these conditions include prevalence of specific cytokines, adhesion to- and co-stimulation by specific stromal cells, and still unknown microenvironmental factors

intrinsic to those lymphoid compartments that favor commitment of B cells to specific immunoglobulin isotypes or T cells to peripheral or mucosal immunity. The distinction between systems that regulate "humoral" versus "cellular" immunity ***(Rocken, M., 1996)*** should not be confused with commitment to "peripheral" or "mucosal immunity". Cellular and humoral immunity is present in both peripheral and mucosal systems but the antibody isotypes and the organ selectivity of the effector T cells may be different Some of the same cell interactions and cytokines involved in controlling cellular or humoral immunity are also involved in triggering peripheral versus mucosal immunity, and vice versa ***(Mosmann, T.R., 1996)***.

**Figure 5: Antigen acquired by different lymphatic tissues**



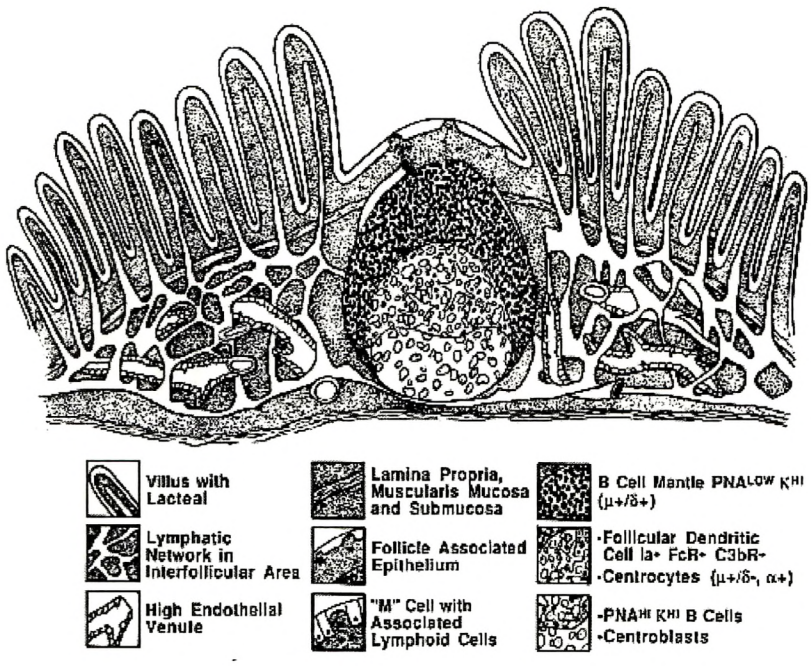



**Figure 6: Peyer's Patches:** The prototypical Mucosal Lymphatic Tissue is the Peyer's patch, which has a unique dome epithelium that is specialized to sample environmental antigens. Peyer's patches contain lymphoid compartments that are analogous to the deep cortex and follicles of lymph nodes, but there are no afferent lymphatics and no medullary cords for local accumulation of plasma cells. Each Peyer's patch contains multiple individual B-cell follicles separated by diffuse lymphoid tissue in interfollicular areas.

All immunoglobulins participate in peripheral or mucosal immunity by binding antigen in a pocket formed by the complementarity-determining regions (CDR) encoded by the immunoglobulin heavy and light chain variable region genes ***(Carayannopoulos, L., 1993)***. The class of the antibody, conferred by the heavy chain constant (C) region genes, determines how it will function and where it will act. Antibody C-region genes are expressed after rearrangement of the selected heavy chain gene and assembly to the already rearranged variable, diversity and joining region genes. Thus IgM, IgD, IgG3, IgG1, IgG2a, IgG2b, IgE and IgA each have heavy chains that control how they participate in immunity, especially with regard to third-party molecular interactions such as Fc receptor binding, activation of the complement system and endosomal transport across mucosal epithelial cells.

**2.1.10.2 Peripheral Immunity and IgG:**

Antibodies of peripheral immunity protect the parenchymal organs and peripheral anatomic sites bathed in tissue fluid and supplied by the blood microvasculature. These antibodies maximize cellular uptake and internalization of antigens. After pathogens have breached the barriers of the skin and/or mucous membranes, antibodies of the IgM and IgG subclasses work in conjunction with the complement system. This collaboration serves to neutralize, injure, aggregate and opsonize the pathogens so that they may be engulfed and destroyed by phagocytes. Except in rare instances where pentameric IgM may be secreted across epithelium. Most circulating antibodies of the IgM and IgG subclasses work in blood, lymph and tissue fluids. They do not normally appear in mucosal secretions ***(Underdown, B.J., 1994).***

**2.1.10.3 Mucosal Immunity and Secretory IgA:**

Antibodies of mucosal immunity function outside the body at luminal surfaces of the moist epithelium lining conjunctiva, nasopharynx, oropharynx, gastrointestinal, respiratory and urogenital tracts and in the ducts or acini of exocrine glands. The principal antibody involved in mucosal immunity is secretory IgA ***(Underdown, B.J., 1994).*** This class of antibody requires the cooperation of two cell types for optimal activity in vivo. One cell makes the IgA and another cell transports it to the gut lumen where it works. The antibody-forming plasma cell releases dimeric IgA, postranslationally associated with J chain. The J chain holds the two IgA molecules together and facilitates binding to the poly-Ig receptor displayed on the abluminal side of epithelial cells. The complex is transported in endosomes to the luminal side of the epithelial cell and released into the secretions. The portion of the poly-Ig receptor retained with secreted IgA is called secretory component.

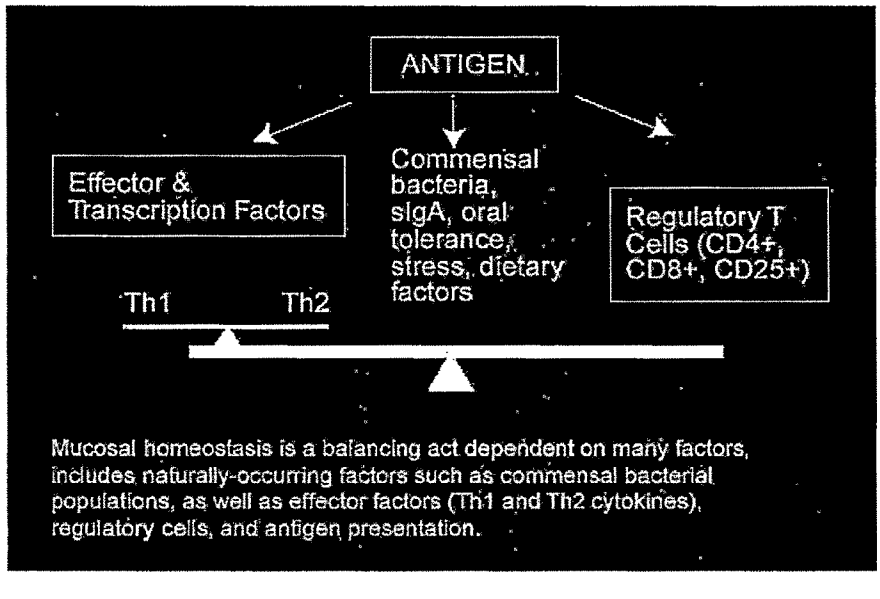
Pathogens adapted to infect mucosae express virulence factors that allow them to adhere, colonize or invade epithelium. Secretory IgA prevents absorption of these viruses, bacteria and toxins by blocking their adhesion while they are still on the external side of the epithelial barrier. This activity is opposite to that of antibodies associated with peripheral immunity. By preventing cellular attachment of the antigen, IgA enables it to be flushed away in the stream of secreted fluids and mucous washing over the epithelial membranes. **(Figure 7).**

IgA may also facilitate transport of pathogens and toxins out of the body by causing them to be conveyed into bile and other exocrine secretions ***(Mazanec, M.B., 1993).***

Antigen-specific IgA has recently been shown to neutralize viral pathogens during transport across "M"cells of Peyers patches, where nondegradative endosomal transport might otherwise deliver a pathogen into the host *(Owen, R.L., 1974; Neutra, M .R., 1994; Mazanec, M .B., 1992).*

IgA is the preponderant antibody manufactured by the body. This escaped appreciation for many years because the blood contains a relatively low concentration of IgA compared to other immunoglobulins. However, 75 percent of the antibody-producing cells in the body make IgA, and most of this IgA is released continuously into gastrointestinal fluid, saliva, tears, urine and other secretions.

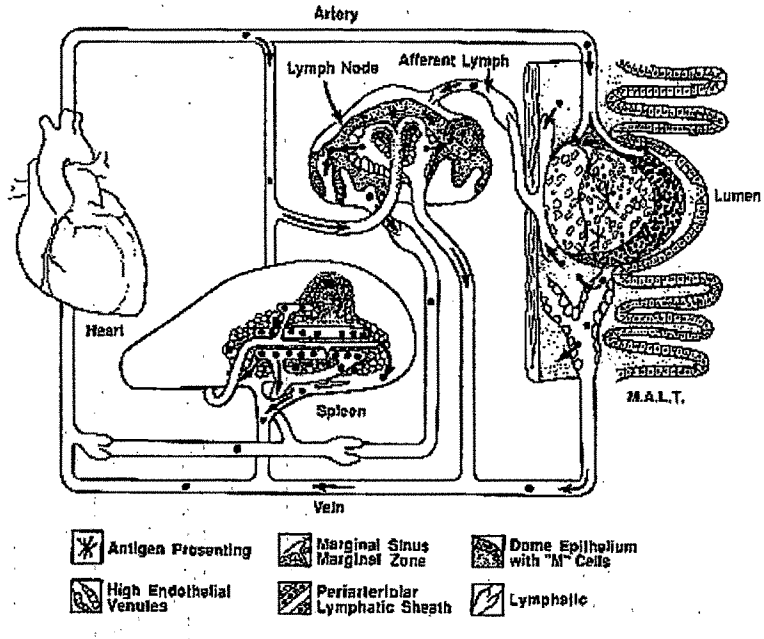
**Figure 7: Mucosal Homeostasis**




### 2.1.11 Compartmentalized Immunity and Lymphocyte Traffic:

Immunity depends upon continuous movement of cells through blood, tissue and lymph **(Anderson, A.O., 1996)**. Lymphoid cells travel to the secondary lymphoid organs of the spleen, lymph nodes and Peyer's patches to encounter antigens acquired from the environment via blood, lymph or across mucous membranes. Where and by which cells antigens are presented to the trafficking cells has a significant influence on the outcome of the immune response with respect to antibody isotype commitment and future homing preference of memory and effector lymphoid cells.**(Figure 8)**

**Figure 8: Recirculation of the lymphocytes**




**This drawing simplifies structures and connections of secondary lymphatic tissues where antigen may most efficiently direct immune responses. Multiple known and unknown factors intrinsic to the microenvironments of lymph node, spleen and mucosa associated lymphatic tissue (here MALT is represented by Peyer's patches) influence whether a "peripheral" or "mucosal" type of response occurs. The drawing also indicates that these tissues are integrated with each other and the rest of the individual through vascular and lymphatic connections and a system of lymphocyte recirculation.**

Recirculation of a precursor pool of uncommitted lymphocytes from the blood into lymph nodes or mucosal lymphatic tissues and then back to the blood again, forms the basis of immuno-surveillance and integration of immune functions across the segregated systems. The magnitude of cell traffic reflected by the number of cells returned to the blood in efferent lymph is enormous. Enough lymphocytes recirculate from lymph to blood to replace the total blood lymphocyte pool from 10 to 48 times every 24 hours. **(Figure 8)**

While naïve lymphocytes appear to randomly access peripheral lymphoid tissues during recirculation, memory lymphocytes selectively return to the tissues where they first were stimulated by antigen. Precursor lymphocytes (that have not seen antigen in a lymphatic tissue or participated in an immune response) enter all tissues, especially secondary lymphatic tissues, such as Peyers patches, peripheral lymph node or spleen, without any organ selective bias. Shortly after activation by antigen, lymphocytes behave like inflammatory cells and avoid returning to secondary lymphoid tissues, preferring to

lodge in skin, gut or inflammatory sites as "acute memory" cells. After maturation occurs, some of these memory cells will follow an organ-selective traffic route determined by the tissue in which that particular lymphocyte encountered the signals to divide differentiate and mature.

If lymphocytes are activated in lymph nodes that drain skin, they become specialized to preferentially migrate into skin and serve the immunological needs of skin *(Mackay, C.R., 1992; Mackay, C.R., 1991)*. Conversely, if lymphocytes are activated in lymphatic tissues, sampling antigens from the gut, they become specialized to preferentially migrate into the gut and serve its immunological needs *(Kimpton, W.G., 1989; Abernethy, N.J., 1991)*.

Some precursor B cells are first activated in spleen or lymph node. In that process, they are programmed to be one of the IgG isotypes (rather than IgA). Daughter cells from this clonal expansion leave the lymph node in the efferent lymph, return to the blood and seed the spleen, bone marrow, sites of inflammation and other lymph nodes as plasma cells and "memory" B lymphoblasts.

Other precursor B cells are first activated in Peyer's patches. Those cells become committed to rearrange their immunoglobulin heavy chain genes to express IgA (rather than IgG isotypes); despite having made this commitment, the switch to IgA is often delayed for several days. This cell leaves in the efferent lymph, passes through the mesenteric lymph node (where it may be subject to immunoregulation by T cells it encounters there) and returns to the blood. These cells selectively migrate to the spleen, which serves as an auxiliary site for clonal expansion before dissemination of the daughter cells via the blood into mucosal lamina propria. This process takes 5-7 days, at least four trips in the blood and two to three cell generations.

**2.1.12 Oral Tolerance and Mucosal Immunity:**

The mucosal immune system is known to be the site of priming for two paradoxically opposite purposes, i.e., tolerance and mucosal immunity. The usual response of the gastrointestinal tract to antigens is tolerance rather than immunity *(Chen, Y., 1995)*. The mechanisms of oral tolerance is still unclear. Tolerance could be primed in Peyer's patches or in the intraepithelial compartment. IEL are good candidates for communicating a toleragenic signal. Epithelial cells can present antigens; but, without important costimulatory molecules, the cells that see this antigen may die or be rendered non-responsive *(Matzinger, P., 1994)*. Tolerance can occur through active suppression

(cells or cytokines that induce non-responsiveness), anergy (live, non-responsive cells), or apoptosis (signal programs cell death) of antigen-reactive cells.

The healthy gastrointestinal tract is bathed in an enormously diverse collection of environmental antigens, yet only certain antigens stimulate active mucosal, peripheral or combined mucosal and peripheral immune responses. Somehow the tissue is able to distinguish pathogens (dangerous) from normal flora (safe) and food antigens (safe) *(Matzinger, P., 1994)*. Inducible chemokines may be the mucosal "danger" signal *(Oppenheim, J.J., 1991; Eckmann, L., 1993)*. Pathogens that bind and/or invade mucosal epithelium cause epithelial cells to release cytokines and chemokines that attract inflammatory and/or immune cells, or cause epithelial cells to express proteins (classical and non-classical MHC) that target antigens for induction of immunity *(Eckmann, L., 1993; Agace,W., 1993; McCormick, B.A.,1993)*. Mucosal antigens that lack co-stimulatory activity are programmed for tolerance *(Kagnoff, M.F., 1996)*. When tolerance results from mucosal immunization, it does not appear to diminish the stimulation of B cells committed to IgA secretion, however. Mucosal tolerance *(Mowat, A.M., 1994)* is most likely responsible for preventing systemic responses to common food antigens.

**2.1.12.1 Abrogation of Oral tolerance**

Induction of simultaneous peripheral and mucosal immunity was explored by many researchers using biodegradable microspheres and enterotoxin-derived, vaccine-delivery systems. Antigens administered orally in biodegradable microspheres composed of poly (lactide/glycolide) copolymer (O'hagan,1993), Polymerised Liposomes (Clark, A.M., 2002) and Chitosan *(van der Luben, I.M., 2003)* or antigens associated with carrier proteins derived from cholera toxin (CT), or *Escherichia coli* heat-labile enterotoxin (LT), induced simultaneous peripheral and mucosal immunity *([a]Elson, C.O., 1984; [b]Elson, C.O. , 1984)*. The same or similar antigens given without these particular vehicles yielded cross regulation. In addition, antigens conjugated with the B-subunits of CT or LT triggered acceptable peripheral and mucosal immune responses that were unattainable when each antigen was given alone *(Dertzbaugh, M.T., 1991; Holmgren, J., 1993; Elson, C.O.,M.T., 1994)*. These results provided evidence that cross regulation could be overcome by using non-toxic and biodegradable vaccine vehicles.

### 2.1.12.2 Microencapsulated Vaccines to overcome Oral Tolerance and Cross Regulation:

After reports of success in enhancing immune responses with adjuvant and particulate antigens, numerous particulates were tested for vaccine enhancing activities. The chemical composition of particles that were tested included polystyrene, latex, poly(methylmethacrylate), polyacrylamide, poly(butyl-2-cyanoacrylate), chitosan, alginate, ethyline-vinyl acetate copolymer, and poly(lactide glycolide) copolymer *(Michalek, S.M., 1994)*. These polymers exhibit a broad range of utility, toxicity and biodegradability.

When vaccine antigen in poly (lactide/glycolide) copolymer was administered orally in heterogeneous sizes, including 10 μm and >1μm sizes, particles of >10 μm diameter were not absorbed in the gut. The larger absorbable particles (diameters >5 to ~10 μm) arrested in Peyer's patches, whereas the smaller particles (<5 to ~1 μm) were disseminated to mesenteric lymph nodes and spleen *(Eldridge, J.H., 1990)*. This bimodal anatomic distribution of particles was accompanied by simultaneous elicitation of IgG and IgA immune responses. Hydrophobicity enhanced uptake of the particles in Peyer's patches and hydrophilicity favored dissemination. The size of the particles appeared to be the critical determining factor that enabled simultaneous induction of peripheral and mucosal immune responses. Similar results were shown by van der Lubben using chitosan as carrier for the oral vaccination against diphtheria, where antigen friendly aqueous microencapsultion was performed.*(van der lubben, I.M., 2003)*

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## 2.2 TETANUS

Tetanus is unique among diseases for which immunization is routinely recommended because it is not communicable. *Clostridium tetani,* the causative agent of tetanus, is widespread in the environment; many animals in addition to humans can harbor and excrete the organism and its spores. When spores of *C. tetani* are introduced into the anaerobic/hypoaerobic conditions found in devitalized tissue or punctures, they germinate to vegetative bacilli that elaborate toxin. The clinical presentation results from the actions of this toxin on the central nervous system (CNS). Many animal species besides humans are susceptible to the disease. ***(Burrows, W., 1959; Wassilak, S. G. F.,2002)***

The word tetanus comes from the Greek 'tetanos'-from the verb 'teino' which means to stretch. The clinical term 'opisthotonus' comprises of two Greek words, opistho and tone which means 'behind' and 'stretching or tightening' respectively indicating thereby a condition in which the muscles of the back are so tightened that the back is arched off the bed and only the head and the buttocks are in contact with the bed. The statistics for tetanus are unreliable and grossly underestimates for in those parts of world where tetanus is common, it is often difficult to obtain reliable data of incidence or mortality. ***(Burrows, W., 1959; Singh, H.,1993)***

### 2.2.1. History of disease and vaccine

- 1884:Carle and Rattone demonstrated that, when the contents of a pustule from a fatal human case were injected into sciatic nerve in a rabbit model, the typical symptoms of tetanus resulted
- 1886: Spore-forming bacilli were observed in the exudate obtained from a human case
- 1889: The spores of *C. tetani* were shown to survive heating and to germinate under anaerobic conditions; injection of pure cultures reproducibly caused the disease in animals
- 1890: Identification and purification of the toxin.
- 1924: Preparations of antibodies derived from horses, became the first means to prevent and treat tetanus leading to preparation of "anatoxin"--chemically inactivated toxin, now termed as toxoid
- 1927: Ramon and Zoeller combined tetanus toxoid with diphtheria toxoid and demonstrated that there was no antigenic competition for immune responses;

they also recognized neonatal tetanus as a disease to be prevented by immunization of pregnant women.

- 1938: Tetanus toxoid became commercially available in the United States
- 1941: US Military began routine prewound prophylactic inoculation
- 1965: WHO standardized the calibration of the potency of tetanus toxoid containing vaccines and established the first international standard for Tetanus Toxoid, adsorbed and plain
- 1974: Expanded program of Immunization launched by the WHO

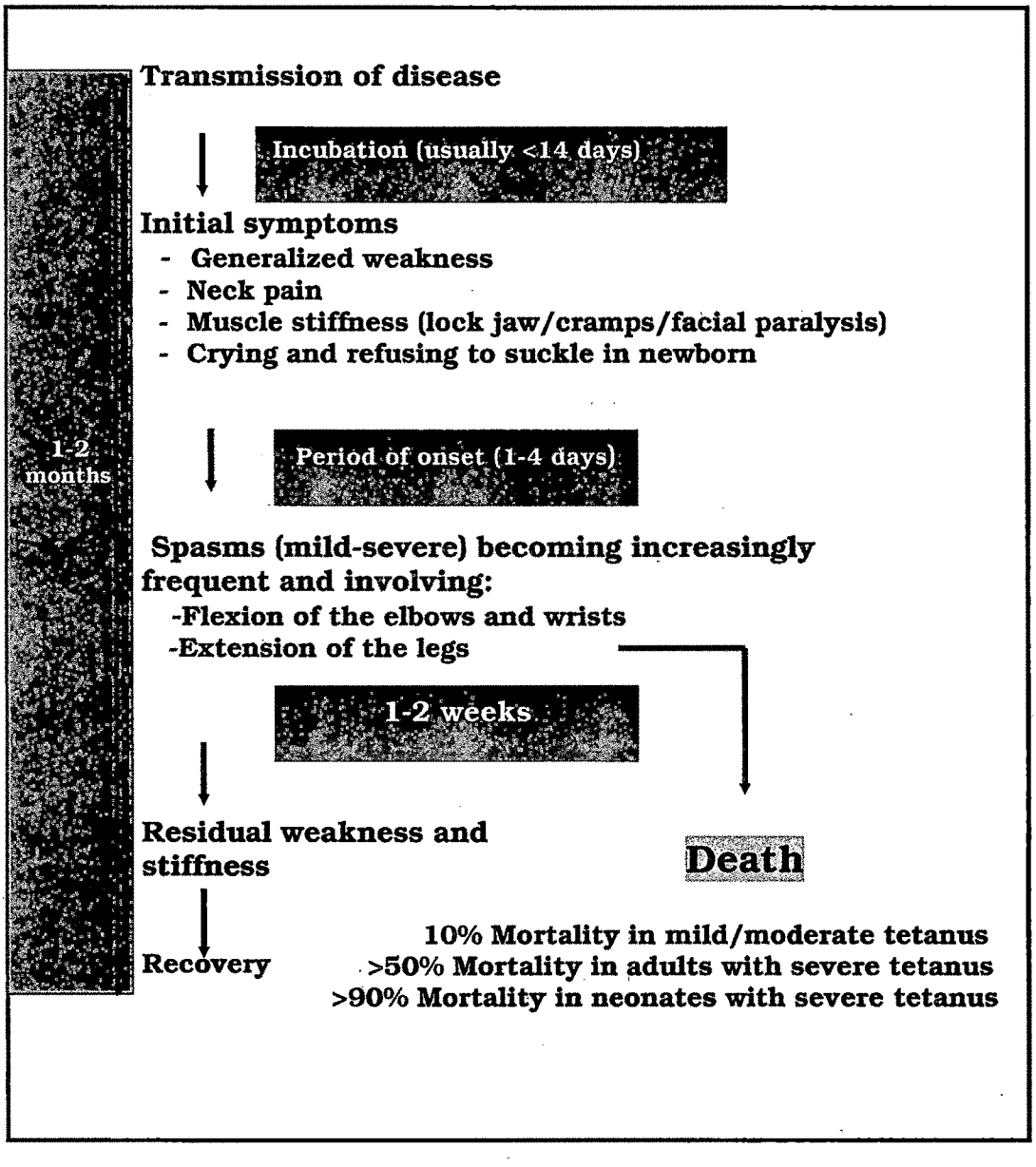
**2.2.2. Clinical Description**

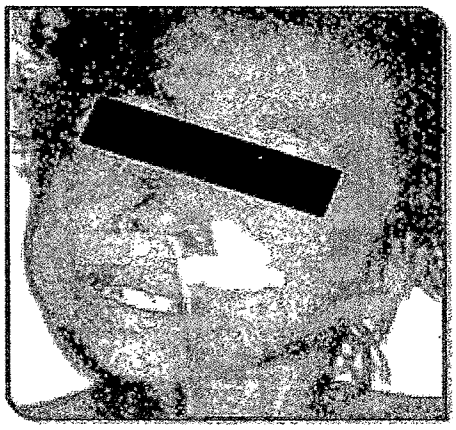
The incubation period for tetanus has been reported to vary 3 days to 3 weeks after inoculation of spores. Three clinical syndromes are associated with tetanus infection: (1) localized, (2) generalized, and (3) cephalic. (***Weinstein, L., 1973***)

More than 80% of cases of tetanus are generalized. The most common initial sign is spasm of the muscles of mastication- trismus, or lockjaw--occurring in more than 50% of the cases. (***Weinstein, L., 1973; Pratt, E.L., 1945***) Trismus associated with spasm of the facial muscles results in a characteristic facial expression-***risus sardonicus***--consisting of raised eyebrows, tight closure of the eyelids, wrinkling of the forehead, and extension of the corners of the mouth laterally. ***(Photo 1 and 2)*** Trismus may be followed by spasm of other muscles in the neck, thorax and back, abdomen, and extremities. Sustained spasm of back muscles can give rise to opisthotonos. Generalized tonic tetanic seizure-like activity (termed tetanospasm), often triggered by mild external stimuli such as sudden noises, consists of sudden painful contraction of all muscle groups resulting in opisthotonos, adduction at the shoulders, flexion of the elbows and wrists, and extension of the legs. Spasm of the glottis can result in immediate death. **(Figure 8)**

Patients exhibit generalized hyperreflexia. Cognitive functions are not overtly affected. Tetanus can be accompanied by severe autonomic nervous system abnormalities, particularly among the elderly and narcotic addicts, that consist of systemic arterial hypertension or hypotension, flushing, diaphoresis, tachycardia, and arrhythmias. (***Kerr, J.H., 1968; Zacks, S.I., 1968; Kanarek, D.J., 1973***)

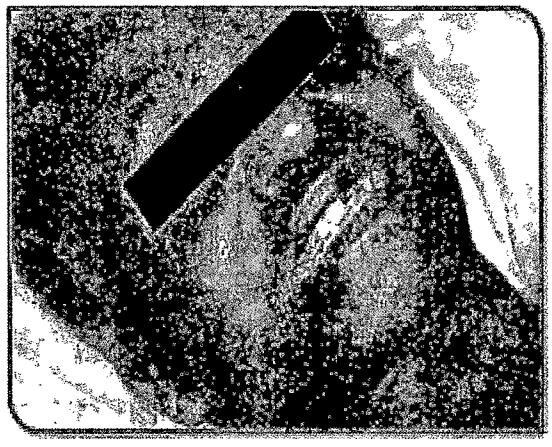
Figure 8: Clinical Manifestations of Tetanus





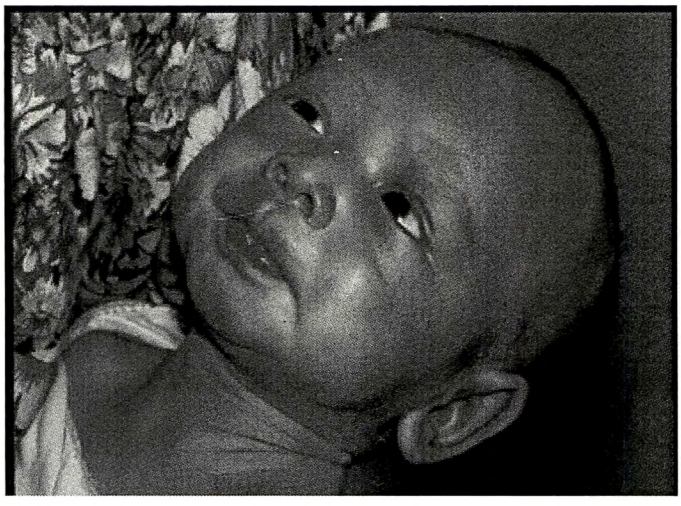
a - Tetanus trismus

b - Tetanus opisthotonos

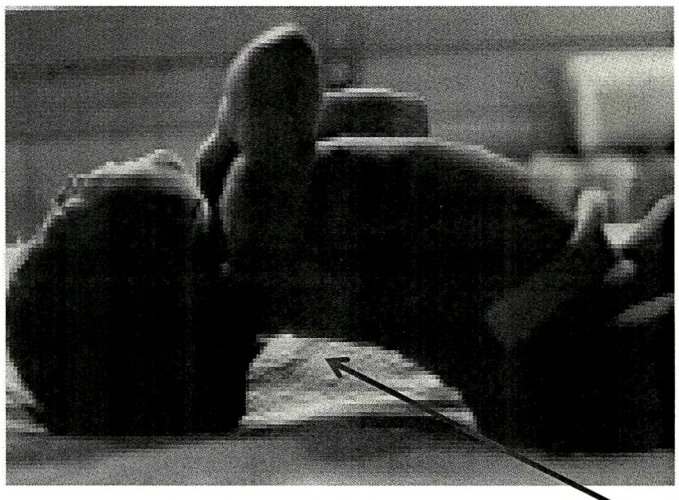


c - Tetanus *risus sardonicas*

**Photo 1: Symptoms of Tetanus**



(a)




(b)

**Photo 2: ( a, b ) Neonatal Tetanus**

Tetanus neonatorum is a form of generalized tetanus occurring in newborn infants, most often as a result of an infected umbilical cord stump. The illness typically begins 3 to 14 days after birth with poor sucking and excessive crying in an infant with normal ability to suck in the first 2 days of life. (***Burrows, W., 1959; Weinstein, L., 1973***) This is followed by variable degrees of trismus, difficulty swallowing, opisthotonos, and other tetanic spasms **(Photo 1 and 2)**.

Death-to-case ratios for reported cases of generalized tetanus have varied from 25% to 70% overall, with fatality ratios in the past approaching 100% at the extremes of age. With modem intensive care, mortality currently can be reduced to 10% to 20%.(***Brauner, J.S., 2002; Trujillo, M.J., 1980; Cook, T.M., 2001***)

Cephalic tetanus is a rare manifestation of the disease generally associated with lesions of the head or face. ***(Weinstein, L., 1973; Jagoda, A., 1988)***

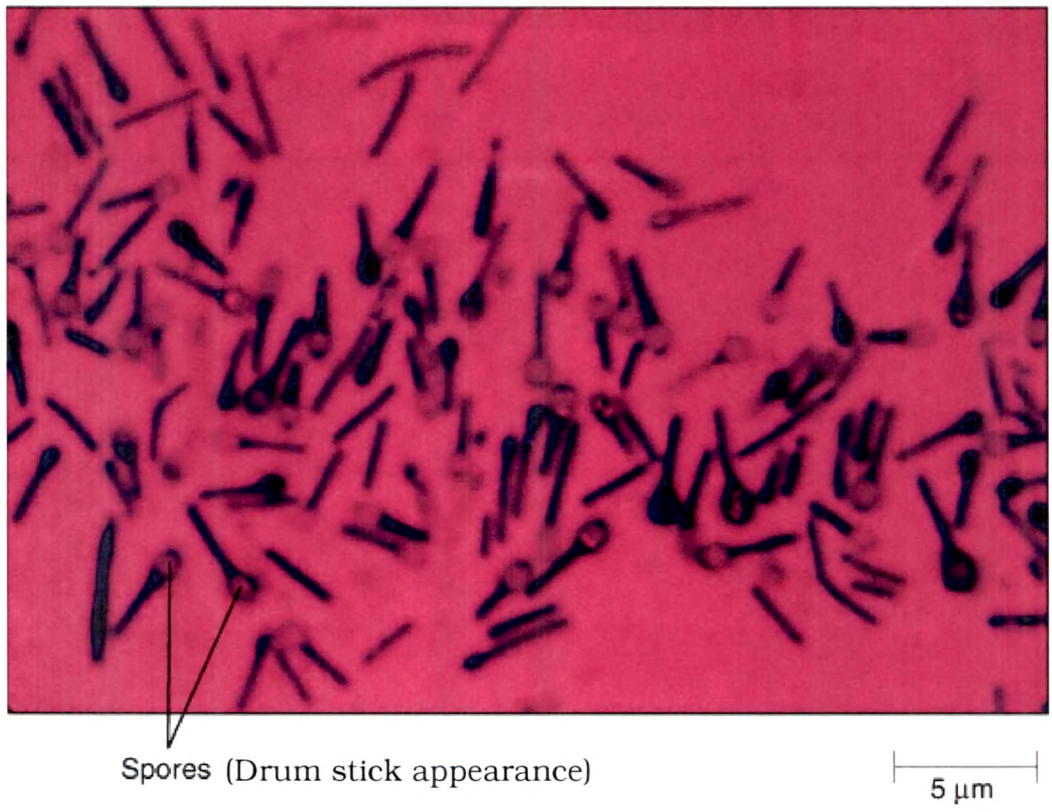
**2.2.3. Bacteriology**

*Clostridium tetani* is a gram-positive, spore-forming, motile, anaerobic bacillus. ***(Hatheway, C.L., 1998; Sanada, I., 1965)*** Typically measuring 0.3 to 0.5µm in width and 2 to 2.5 µm in length. **(Photo 3)** Flagellae are attached bilaterally on non-spore-forming bacteria. With sporulation, *C. tetani* takes on the more characteristic drumstick-like appearance. Spores usually form in the terminal position. *C. tetani* is considered a strict anaerobe that grows optimally at 33°C to 37°C. If not exposed to sunlight, the spores can persist in soil for months to years. ***(Weinstein, L., 1973)*** The most common source of environmental exposure to *C. tetani* bacilli and spores is the soil. Animals, both herbivores and omnivores, can carry *C. tetani* bacilli and spores in their intestines and readily disseminate the organism in their feces.

**2.2.3.1 Pathogenesis:**

*C.tetani* fails to produce invasive cellulitis when present all alone in the human body. Other factors, which stimulate the production of pathogenicity, are not known. It is hypothesized that other bacteria present in the lesion play an important role in reducing the oxidation-reduction potential at the site of injury and only then tetanus bacilli produce their soluble products. 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.



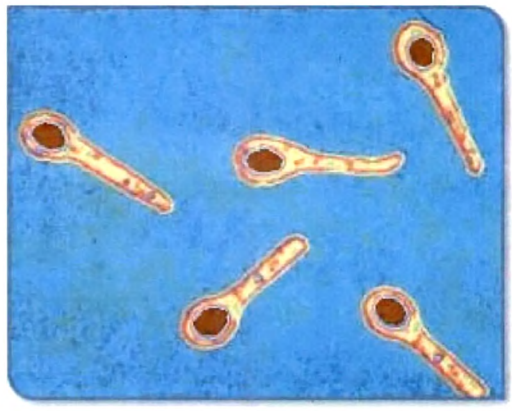



**Photo 3:** *Clostridium tetani*

When released in culture medium, the prototoxin is cleaved by proteases into light (toxic moiety) and heavy (binding) chains with molecular weights of 50,000 and 100,000,

respectively, containing two disulfide bonds-one between chains and one internal to the heavy chain.*(Matsuda, M., 1975; Bizzini, B., 1984; Robinson, J.P., 1982; Schiavo, G., 1990)* The C-terminal end of the heavy chain (fragment C) is the moiety that binds to gangliosides.*(Ahnert-Hilger,G., 1990)* The light chain is an endopeptidase that cleaves a membrane protein of synaptic vesicles.**(Figure 9)**Toxin production appears to be under the control of a plasmid *(Laird, W.J., 1980; Finn, L.W. Jr., 1984)*

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)*

Transport of toxin from the injured site into the CNS is complex.

**2.2.3.2 Mode of action-** The molecular basis for the action of tetanospasmin is not known. The site of action is the synaptosomes (nipped-off nerve endings) that are high in toxin fixing capacity. This binding of toxin takes place because of gangliosides in synaptic membranes. The gangliosides are water soluble mucolipids containing residues of stearic acid, glucose, galactose and sialic acid. The toxin specifically affects the synaptic junction of nerves by preventing the inhibition or erasing of nerve impulses once they have crossed the synaptic junctions. The nerve continues to send impulses, a condition that results in spasmodic contraction (tetany) of the involved muscles.

The action of tetanus toxin proceeds via four steps:

1. Extracellular binding

2. Initial translocation across cell membranes

3. Intra-axonal and trans-synaptic transport to site of action

4. Intracellular poisoning of specific neurotransmitter release processes.

Toxin is initially bound to nerve terminal membranes at neuromuscular junctions, then is internalized and migrates to its ultimate sites of action in the central nervous system

primarily via retrograde axonal transport. Once toxin has reached the spinal cord, it ascends within it. Some toxin may also travel via blood, particularly in generalized tetanus with a short incubation period. However, since toxin cannot cross the blood brain barrier, it is less likely to enter the spinal cord by this route.**(Figure 10)** ***(Rappuoli R.,1997)***

A component of nerve tissue exhibiting receptor-like features for tetanus toxin has been identified. When tetanus toxin is mixed with brain emulsion or with subcellular fractions rich in synaptosome membranes, a comparatively large dose can be injected into a susceptible animal without causing damage. This is known as *Wassermann-Takaki phenomenon.* The substances responsible for binding of toxin are gangliosides (GD1b and GT1b), complex glycolipids containing N-acetylneuraminic acid (sialic acid). Tetanus toxin binds to isolated neural gangliosides or to tissues (or tissue fractions) containing surface gangliosides. Binding is prevented in vitro by treatment with neuraminidase, which cleaves sialic acid residues from gangliosides. ***(Rappuoli R.,1997)***

As with botulinum toxin, it seems that the *translocation* of tetanus toxin at the neuromuscular junction occurs by receptor mediated endocytosis. The B fragment of the tetanus toxin forms channels in the artificial phospholipid vesicles at low pH. The hydrophobic region of the fragment may cause pores in vivo thus allowing transport of toxic L chain into the cytosol. ***(Singh, H., 1993; Rappuoli R.,1997)***

Intra-axonal retrograde transport of tetanus toxin occurs within smooth muscles and when these reach the dendrites of the neurons, the toxin is transferred trans-synaptically to its

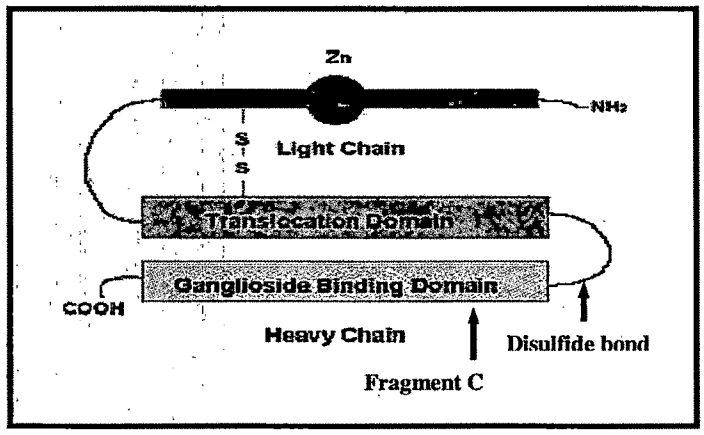
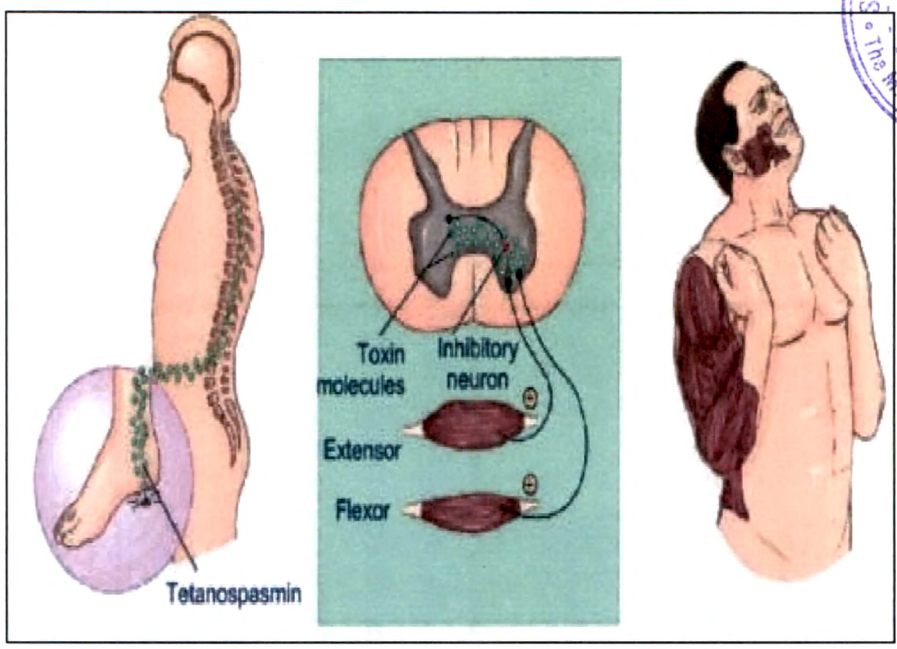



**Figure 9: Structure of Tetanus Toxin (Tetanospasmin)**



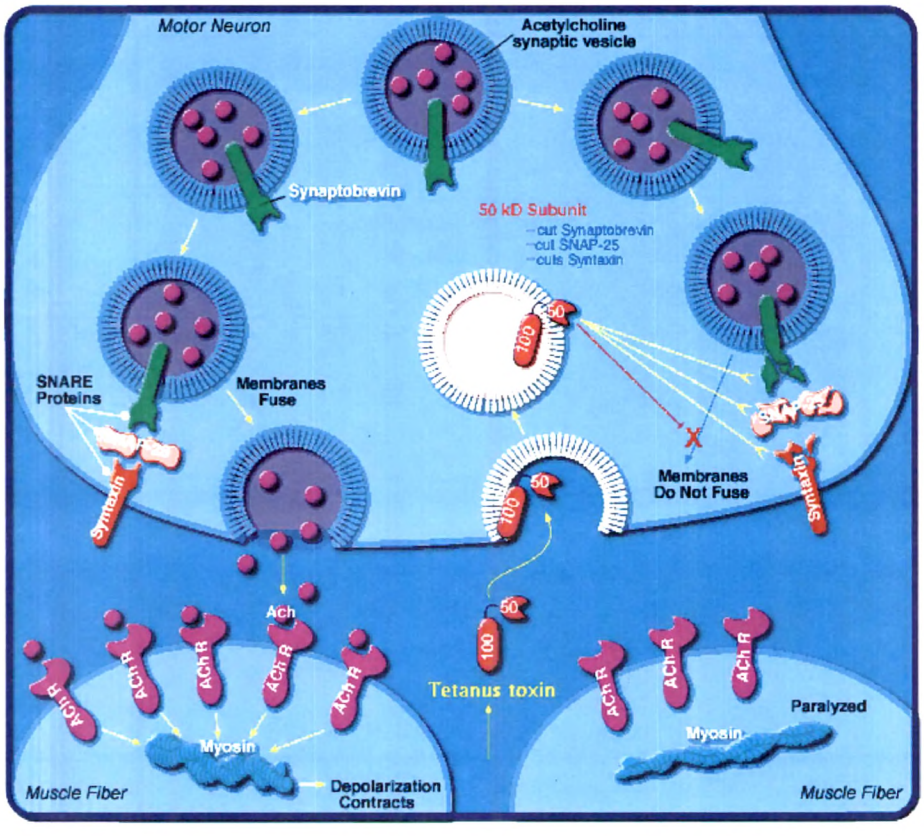




Figure 10: Tetanus toxin : Mode of action

site of action, the abutting nerve terminals. The spasmogenic effect of the toxin may be due to its action on polysynaptic reflexes involving two types of inhibiting interneurons in the spinal cord. It blocks the normal inhibition of spinal motor neurons following afferent impulses postsynaptically by preventing the release of inhibitory transmitters glycine and gamma-amino butyric acid. The resulting sensitivity to excitatory impulses, unchecked by inhibitory mechanisms, produces the generalised muscular spasms characteristic of tetanus. The precise biochemical mechanism by which tetanus toxin blocks the release of neurotransmitters is not known. Tetanus toxin does not cross the blood-brain barrier *(Rappuoli R., 1997; Abel, J.J., 1938)*; neuronal transport is the sole means of entry into the CNS. *(Weinstein, L., 1973; Friedemann, U., 1939; Bizzini, B., 1979)* After it gains entry at neuromuscular junctions by binding to gangliosides,*(Fedinec, A.A., 1985)* toxin

proceeds up the nerve by intra-axonal transport to the ventral horns of the spinal cord or motor nuclei of the cranial nerves.*(Price, D.L., 1975; Parton, R.G., 1987)* The disease can progress clinically despite use of parenteral antitoxin. Tetanospasmin can act at the peripheral motor end plates, the spinal cord, the brain, and the sympathetic nervous system.*(Kerr, J.H., 1968; Zacks, S.I., 1968; Kanarek, D.J., 1973; Brooks, V.B., 1955; Brooks, V.B., 1962)* The toxic moiety cleaves the synaptic vesicle membrane protein synaptobrevin and causes disinhibition of spinal cord reflex arcs by interfering with release of the neurotransmitters glycine and γ-aminobutyric acid (GABA) from presynaptic inhibitory fibers.*(Weinstein, L., 1973; Brooks, V.B., 1955; Brooks, V.B., 1962; Cornille, F., 1997)* Once inhibition is blocked, excitatory reflexes multiply unchecked, causing the tetanic spasms or, at the cerebral level, convulsions.

**2.2.3.3 Treatment**

The purpose of tetanus therapy is (1) to prevent additional circulating toxin from reaching the CNS, (2) to prevent further toxin production by eliminating the organism, and (3) to give supportive care for the duration of the illness. Human tetanus immune globulin (TIG) should be given at the time of diagnosis to neutralize circulating toxin before it reaches the nervous system. *(Weinstein, L., 1973; Bleck, T.P., 2000)* A dose range of 3000 to 6000 units given intramuscularly in a single administration. Equine antitoxin can be given intravenously but is associated with serious allergic side effects such as anaphylaxis and serum sickness. *(Moynihan, N.H., 1955; Merson, M.H., 1974)* Following

intramuscular TIG administration, peak serum levels of antitoxin are achieved by 48 to 72 hours. *(Lee, D.C., 1992)*

All suspected tetanus patients should start or complete a primary series of vaccination with tetanus toxoid, or receive a booster dose, at the time of diagnosis.

Continuing production of tetanus toxin should be prevented by appropriate antimicrobial therapy and surgical drainage or debridement. Procaine penicillin, 1.2 million units daily, or aqueous crystalline penicillin G, 4 million units daily divided every 6 hours, was administered for 5 to 10 days to kill the vegetative form of the organism. *(Weinstein, L., 1973)* Metronidazole (500 mg every 6 hours either intravenously or orally), Erythromycin, Tetracycline, and Clindamycin are acceptable alternatives.

Experimental work with benzodiazepines such as diazepam demonstrated that the $GABA_A$ agonist action of these agents indirectly counteracts the effects of the toxin. *(Bleck, T.P., 1997)* Intravenous diazepam is generally given in doses of 0.5 to 15 mg / kg /day. *(Tekur, U., 1983)*

### 2.2.4. Epidemiology

#### 2.2.4.1 Incidence and Descriptive Epidemiology of Non-neonatal Tetanus

Although tetanus morbidity and mortality has dramatically decreased in developed countries, it remains a major public health problem in most developing countries. The magnitude of the disease is directly proportional to socioeconomic and ecological factors. Large numbers of cases are reported from Africa, Asia and Latin American countries than Europe or the USA. The incidence of tetanus is high in poor countries with warm and humid environment and where manure soil is likely to contaminate the wound. It has been estimated tetanus kills on average 140 times more individuals in poor developing countries than in rich, developed countries. Tetanus incidence is higher by a factor of 5-8 times in rural than in urban settings. It has been probably the fourth commonest cause of death in rural India. 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 in midwifery.

It has been estimated that approximately 50% of neonatal death and 25% of infant deaths throughout the world are due to tetanus. Current estimates of global-mortality are almost one million per year. Of these 8,80,000 death are due to neonatal tetanus and

1,20,000 to 3,00,000 (average 2,00,000) deaths from non-neonatal tetanus. Another group, which is now emerging as more prone to tetanus, is the narcotic addicts. Of 142 cases in New York, no fewer than 102 were drug addicts.

A sample survey conducted in 1981 in India shows that approximately 0.25 million infants die of neonatal tetanus every year. The reported incidence varied from 10 to 20 per thousand live births. Additionally over 50,000 cases of tetanus occur annually in children and adults. And all this inspite of the fact that an economically effective and safe prophylactic agent has been available for more than 50 years!

**2.2.4.2 Incidence and Descriptive Epidemiology of Neonatal Tetanus**

Neonatal tetanus cases often are associated with nonsterile conditions of childbirth, with delivery personnel untrained in sterile care of the umbilical cord and stump or not adequately washing their hands, *(Leroy, O., 1991)* with traditional surgeries *(Bennett, J., 1999)* and particularly with births followed by unhygienic cultural rituals involving the umbilical stump such as application of herbs, clarified butter, or animal dung. *(Leroy, O., 1991; Hamid, E.D., 1985)* Unless their mothers have received two or more doses of tetanus toxoid, newborns are susceptible. The magnitude of the occurrence of neonatal tetanus is particularly important given a death rate of more than 95% without specific therapy and 10% to 90% with therapy, depending on the intensity of supportive care. *(Hamid, E.D., 1985; Gupta, S.M., 1979)*

In the United States, neonatal tetanus deaths during the 20th century became progressively less common before tetanus toxoid was used widely in women, perhaps because of improvements in puerperal hygiene. Deaths from tetanus among infants declined from 0.64 per 1000 live births in 1900 to 0.07 in 1930 and to 0.01 by the 1960s. *(Hinman, A.R., 1987)*

**Figure 11** shows the graph of Global annual roprted incidnce of total Tetanus and DTP3 coverage for the year 1980-2003. The coverage is up to 80% and reported incidences are below 20,000 for the year 2003. **Figure 12** shows the graph of Global annual roprted incidnce of Neonatal Tetanus (NT) and TT2+ coverage for the year 1980-2003. and protection at Birth coverage for the year 2003 only The coverage is up to 50% and reported incidences are below 10,000 for the year 2003. **Figure 13** shows the reoprted Neonatal Tetanus cases in year 1990 and year 2003, 1 dot = 10 NT cases. The number is

reduced to 8,997 in 2003 from 25,293 cases in 1990 due to increased WHO vaccination coverage. Most of the cases are from Asian and African countries.

**Figure 11: Total tetanus global annual reported incidence and DTP3 coverage, 1980-2003**

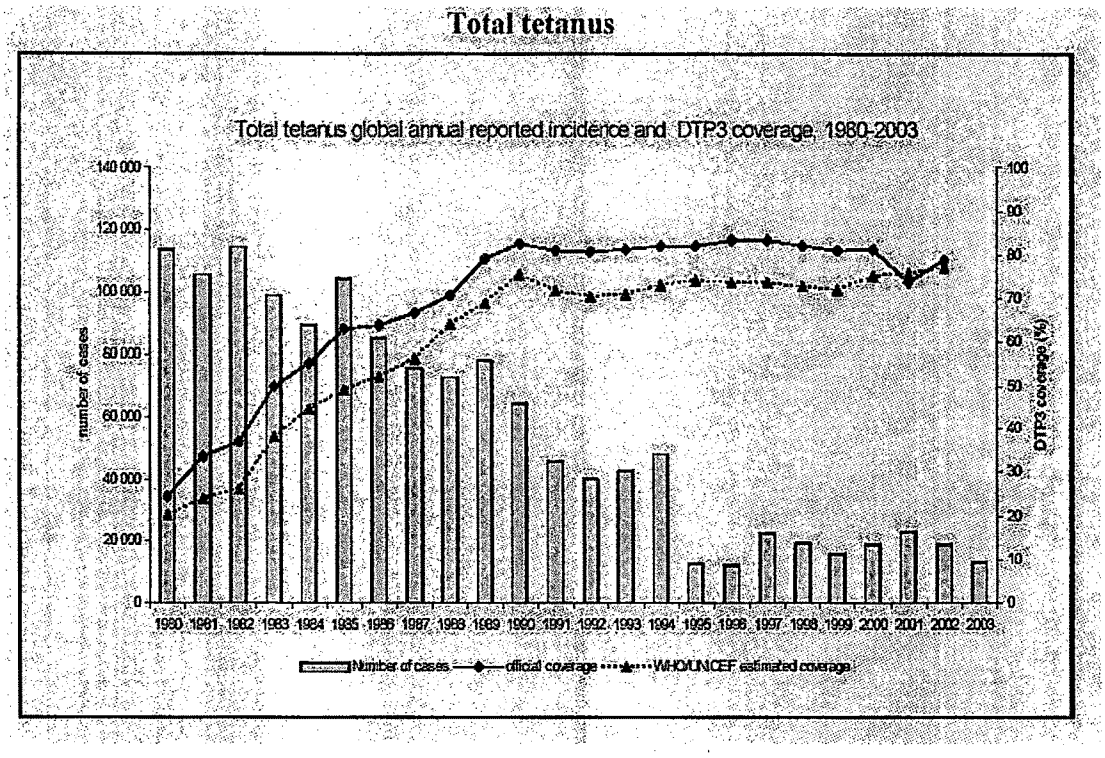
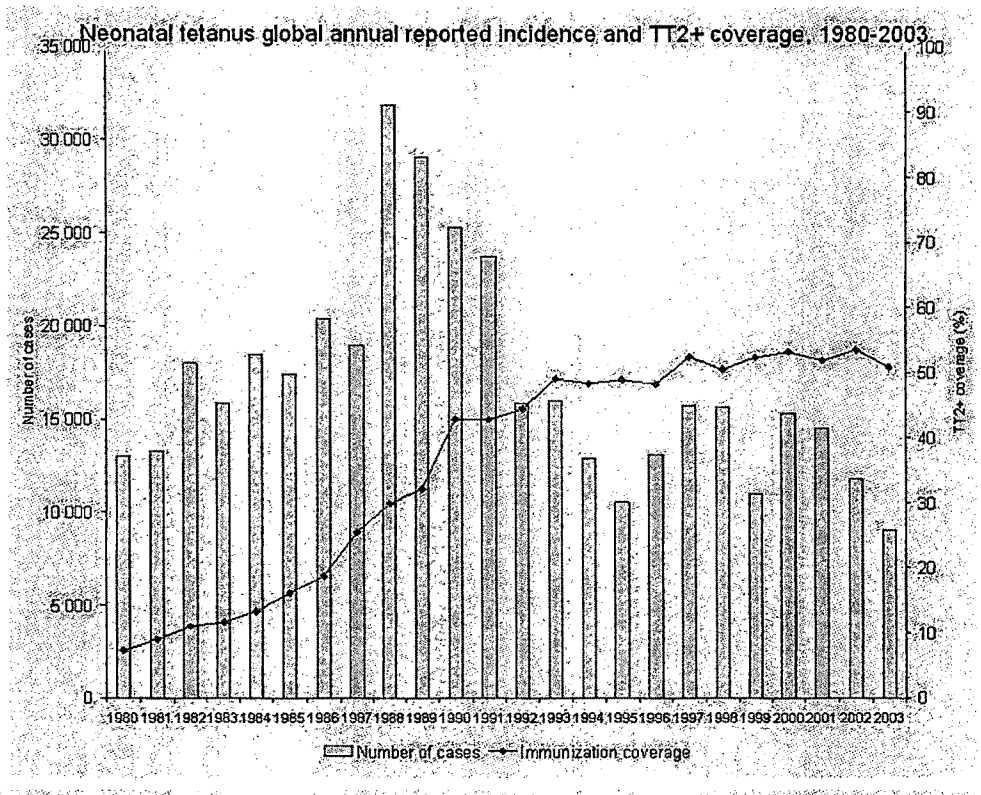


**Figure 12: Neonatal tetanus Global annual incidence and TT2+ coverage, 1980-2003 and protection at Birth coverage for 2003 only.**



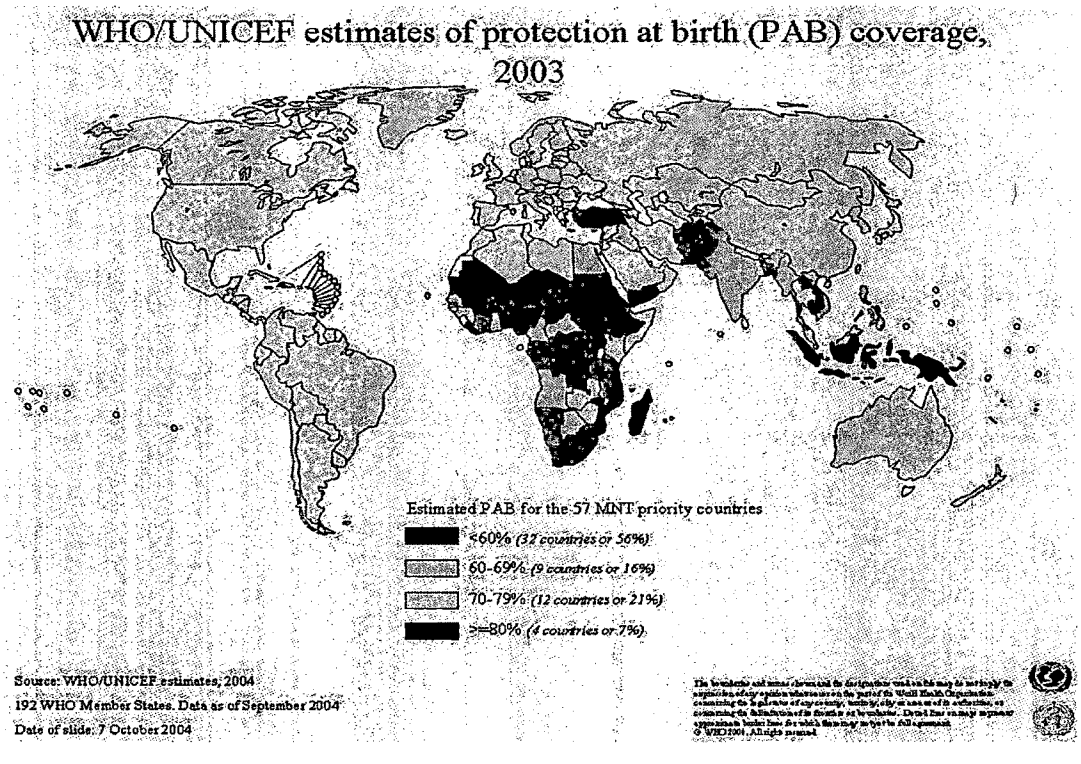
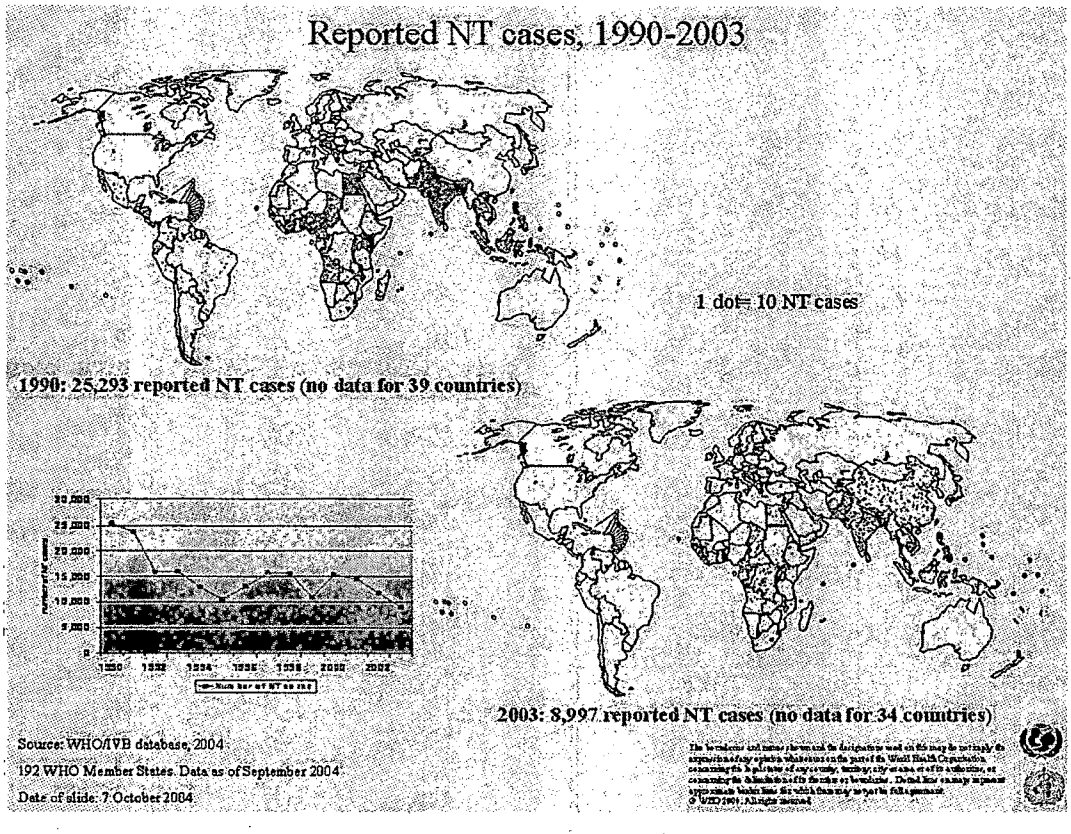
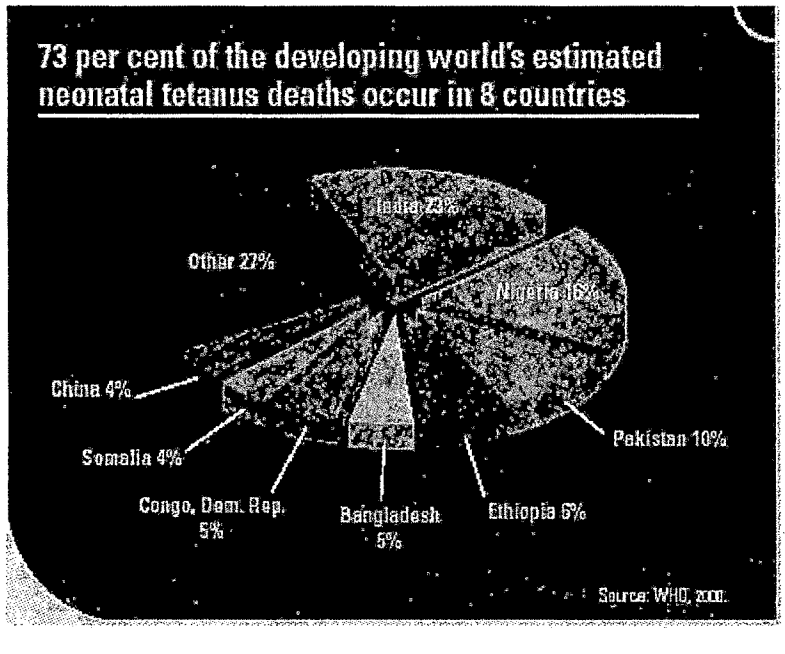



**Figure 13: Reoprted Neonatal Tetanus cases in year 1990 and year 2003: Impact of WHO vaccination coverage.**



### 2.2.5. Passive Immunization and Correlation of Serum Antitoxin with Protection

Despite improvements in the quality of equine antitoxin, the passive immunity it confers is of limited duration. The half-life of refined equine antitoxin in humans is less than 2 weeks and may be considerably less in previously sensitized individuals.

TIG was introduced in the early 1960s and was found to have a fairly constant half-life of 28 days in humans.255 TIG is produced by cold-ethanol fractionation of the plasma of hyperimmunized adults. It is distributed in 1-mL vials with 250 IU/mL

The minimum level of tetanus antitoxin needed to ensure protection against tetanus is generally accepted to be 0.01 IU/mL as measured by in vivo neutralization assay. ***(McComb, J. A., 1964; Smith, J.W.G., 1969; MacLennan, R., 1965).*** This level is based primarily on animal studies of the protective effects of active and passive immunization.

### 2.2.6. Active Immunization: Toxoid

#### 2.2.6.1 Tetanus Toxoid Production and quality control of tetanus toxoid

Tetanus toxoid, just like diphtheria toxoid, was produced till 1950's by exactly the same technology which was adopted by Ramon in 1924. This resulted into formation of crude toxoids which were although effective, had problems of low purity and association with adverse effects. Till 1950s, *C.tetani* was grown in complex medium consisting of digested meat. The rich protein content of this medium made it difficult to subsequently purify the toxin and presence of high levels of foreign proteins in the crude preparations resulted into higher incidence of adverse effects in man. Furthermore, cultures were grown in small vessels, which yielded heterogenous lots of toxin.

Introduction of fermentor technology and a semisynthetic culture medium (Modified Mueller and Miller medium) have brought about significant changes in industrial production of tetanus toxoid as well as augmented the purity of the preparation.

The tetanus toxin is produced by growing a standard hypertoxigenic strain of *C.tetani.* Usually Harvard strain of this organism is preferred which originated from New York State Department of Health and gives consistently high yields of tetanus toxin. This strain is grown in modified Mueller and Miller medium in a fermentor for about one week during which period the fermentor is flushed with air. The bacteria lyse and release tetanus toxin into supernatant. The average yield obtained from these cultures is about 60-80 Lf/ml (1 LF = 2-2.5 μg of tetanus toxin). The culture is filtered and the filtrate

containing the toxin is detoxified by adding 40% formalin to give a final concentration of about, 0.5%. The pH is adjusted to 7.6 with sodium carbonate or bicarbonate solution and the containers are incubated at 37°C for 4 weeks, pH being adjusted every week.

During the detoxification process, formaldehyde reacts with toxin molecules. The first reaction involves the ε amino groups of lysine. This reaction is rapid and fully reversible. The second reaction is slow and inolves the reaction of one of the unstable products from the first; reaction with a second molecule containing an amino group, an imidazole ring or a phenol ring. The final result of the formaldehyde treatment of diphtheria and tetanus toxins involves a cross-linkage between an ε group of lysine and a second amino group, a histidine and a tyrosine or tryptophan through a stable methylene bridge. These reactions can occur between amino acids of same toxin molecule, resulting in internal cross-linking of the protein and between two toxin molecules resulting in dimerization. A significant achievement has been made in the past decade by the substitution of formaldehyde with glutaraldehyde for the detoxification of toxin. Tetanus toxin is completely and irreversibly inactivated by glutaraldehyde at room temperature within 10 minutes. This toxoid has been designated as POLAN-T because of the polymerised nature of the antigen obtained. Its immunogenicity has been enhanced by adsorbing onto calcium phosphate. Large numbers of infants have been successfully immunized with POLAN-T in France without any significant adverse effects.

The bulk purified tetanus toxoid is prepared either from a single harvest or from a pool of harvests. It is sterile. A preservative, other than phenol, can be added. Each bulk toxoid is tested for bacterial and mycotic sterility. The specific toxicity of the toxoid for the presence of tetanus toxin is tested in guinea pigs, which are injected subcutaneously with 1 ml of a dilution of purified toxoid containing at least 500 Lf of toxoid. The bulk toxoid passes the test if no guinea pig shows symptoms of specific paralysis or any other sign of tetanus within 21 days of injection and if at least 80% of the animals survive the test period. Reversion to toxicity is tested after storing the toxoid at 37°C for 6 weeks and by animal inoculation tests.

Each bulk purified toxoid is tested for antigenic purity by determining the Lf value and the concentration of protein (nondialysable) nitrogen. The bulk passes the test if it contains no fewer than 1000 Lf per mg of protein nitrogen.

Certain control tests are performed on the final bulk. The number of Lf in a single human dose shall not exceed 25 Lf if more than one dose is recommended for primary

immunization. A suitable antimicrobial preservative, other than phenol, may be added if the vaccine is to be filled in multi-dose containers. Aluminum or calcium compounds are generally used as mineral carriers (adjuvants). The concentration of aluminum shall not exceed 1.25 mg and that of calcium 1.3 mg per single human dose. The sterility and specific toxicity of the final bulk is tested on the same lines as is done for bulk toxoid.

The immunizing potency of each final bulk is determined by comparison with an appropriate reference material. The test involves inoculation of guinea pigs or mice with appropriate doses or dilutions of both final products and the reference material. The potency of the tetanus toxoid used for immunization of children shall not be less than 40 IU per human dose. In the final bulk, the amount of residual formaldehyde shall not be more than 0.2 gram/litre and the pH of the final bulk shall be between 6.0 and 7.0.

The quality control tests on the final product shall include tests for identity, sterility, potency, innocuity, adjuvant and preservative content as well as pH. The test for innocuity is done by injecting by the intraperitoneal route one human dose into each of 5 mice and two guinea pigs. The final product is considered innocuous if the animals survive for at least seven days without showing significant signs of toxicity. The tetanus adsorbed vaccine is not to be frozen and remains stable at temperatures between 2 to 8°C. *(Singh, H., 1993)*

**2.2.6.2 Dosage and Route**

As with other inactivated vaccines and toxoids, the immunologic response to tetanus toxoid requires more than one dose to confer protection and persisting immunity. *(McCarroll, J.R., 1962; Newell, K.W., 1971)*

The adsorbed toxoid is administered intramuscularly; the fluid TT preparation can be given subcutaneously. The toxoid content of commercial products is assessed by flocculation with standard antitoxin and measured in Lf units [the limits of flocculation (Lf) test as a surrogate measure of potency. Manufacturers of tetanus toxoid indicate the quantity of toxoid in preparations by Lf content and the purity by Lf per milligram of protein nitrogen (pure toxoid has 3000 Lf/mg) *(Bizzini, B., 1985)* This measure of toxoid protein content does not necessarily correspond to immunogenicity as measured by potency in guinea pigs. Adsorbed products available in the United States have a content of 2 to 10 Lf/dose; the fluid TT contains 4 Lf. Potency is determined by animal bioassays.

### 2.2.6.3 Available Preparations

DTaP and DT are combinations used in infants and children younger than 7 years. Universal use of DTP/DTaP in infancy and childhood is recommended unless there are contraindications to pertussis vaccine. Td is used in persons 7 years of age and older because it contains less diphtheria toxoid (2 Lf or less) than the pediatric preparation (DT; more than 10Lf). Single-antigen tetanus toxoid is also available for use in persons 7 years of age and older either as an adsorbed or fluid preparation.

Many tetanus toxoid adsorbed preparations are available with various precipitating salt adjuvants like aluminum hydroxide, aluminum potassium sulfate, or aluminum phosphate. Adsorbed toxoid is preferred because it confers protective levels of antitoxin for a longer time than fluid toxoid ***(MacLennan, R., 1973; Gottlieb, S., 1964)***

#### 2.2.6.3.1 Constituents

According to WHO standards, the final product should contain 0.5% formaldehyde or less. In the United States, minimum requirements stipulate residual formaldehyde content of 0.02% or less. In adsorbed products, precipitating calcium or aluminum salt adjuvants are present. A single human dose must contain less than 1.25 mg of aluminum. The preservative used is thimerosal to a final concentration up to 0.1%, but more typically 0.01%. Recently, phenoxyethanol has been used as an alternative to thimerosal in preparations for children, usually with a final concentration of 0.01 %.

#### 2.2.6.3.2 Stability

Preparations should be stored at 2°C to 8°C and generally have a 2- or 3-year expiration date. Higher ambient temperatures should be avoided, particularly for periods longer than 7 days. Tetanus toxoid exposed to 60°C is destroyed in 3 to 5 hours. Freezing also can reduce potency, particularly when the toxoid is adsorbed. ***(Weinstein, L., 1973; WHO, 1980)***

### 2.2.6.4 Results of Active Immunization

Active immunization confers immunity to tetanus by stimulating production of serum antitoxin. Primary immunization with tetanus toxoid also induces cellular immune responses (T-helper type 1 [Th1] or type IV hypersensitivity) in 74% to 90% of recipients. The gold standard for assessing a serologic immune response to tetanus toxoid is the toxin neutralization test, which measures biologically active antitoxin in serum. ***(Newell, K.W., 1971; Barile, M.F., 1970)*** Neutralization tests are performed in mice injected with

serial dilutions of test serum that have been pre incubated with a lethal dose of tetanus toxin and standardized to a reference serum specimen. Toxin neutralization tests detect antitoxin titers as low as 0.001 IU/mL. These assays are believed to be most reliable because they assess actual neutralization in a living host. Because in vivo neutralization tests are time consuming and expensive, a variety of in vitro serologic tests have been developed. Among these are passive hemagglutination (PHA), ELISA, radioimmunoassays, immunofluorescent assays, latex agglutination, and a variety of methods using agar gel precipitation. Most in vitro assays have the important limitation that they do not discriminate between biologically active antibody and non-neutralizing antibody, leading to a lack of specificity that is most apparent at low antibody titers. ***(Galazka, A.M., 1993)*** PHA and ELISA are currently the most commonly used in vitro techniques to determine tetanus antitoxin levels.

**2.2.6.5 Indications for Use**

Because of the success of active immunization in the military, wide *C. tetani* spore distribution, the high death-to-case ratio of tetanus, and frequent reactions with and incomplete efficacy of equine antitoxin, ***(Press, E., 1948)*** routine inoculation in childhood was recommended in 1944 by the American Academy of Pediatrics (AAP). Advisory groups in the United States, including the Advisory Committee on Immunization Practices (ACIP) have recommended that all people receive three or more doses of tetanus toxoid in the appropriate combination based on age, followed by routine booster doses every 10 years.

**2.2.6.6 Administration to Infants and Children**

The recommended schedule for routine immunization is given in **Table 3 and 4.** WHO-recommended schedule for the developing world of DTP at 6, 10 and 14 weeks of age is being used in many countries in Europe and elsewhere. After two- or three-dose primary schedules during infancy, antibody levels tend to wane ***(Di Sant' Agnese, P.A., 1949; Ramsay, M.E.B., 1993)*** Although children who receive two or three doses of vaccine at 1- or 2- month intervals rarely have lost protective levels of antibody 1 year after the last dose, reinforcing doses at this time lead to high levels of antitoxin production and long-term immunity, generally exceeding 10 years. ***(Gottlieb, S., 1964; Di Sant' Agnese, P.A., 1949; Barkin, R.M., 1984; Barkin, R.M., 1985; Kutukculer, N., 1996; Ramsay, M.E.B., 1993)***

**Table 3: National Immunization Schedule of Vaccination**

| Age | Vaccines |
|---|---|
| Pregnant women | TT (2 doses at 4 weeks interval) |
| Birth | BCG, OPV - 0 |
| 6 weeks | DTP – 1, OPV –1 |
| 10 weeks | DTP –2, OPV –2 |
| 14 weeks | DTP –3, OPV –3 |
| 9 months | Measles |
| 16 –18 months | DTP booster, OPV - 4 |
| 5 years | DT |
| 10 years | TT |
| 16 years | TT |
| Onwards | Every ten years Td |

**Table 4: Tetanus Toxoid: Immunization Schedule for Women of Child bearing Age**

| Dose | Administration | Protection (Approximately) |
|---|---|---|
| **TT1** | As early as possible during pregnancy | None |
| **TT2** | At least four weeks after TT1 | 1-3 years |
| **TT3** | At least six months after TT2 | 5 years |
| **TT4** | At least one year after TT3 or during subsequent pregnancy | 10 years |
| **TT5** | At least one year after TT 4 or during subsequent pregnancy | A women's immunity and her ability to confer short-term immunity to her newborn will last throughout her reproductive life |

**2.2.6.7 Administration to Adults**

The immune response to tetanus toxoid appears to decrease with increasing age. ***(Myers, M.G., 1982)*** Despite the decrease in immunogenicity, the majorities of adult vaccines achieve and maintain protective levels of antitoxin for many years. ***(McCarroll, J.R., 1962; Gottlieb, S., 1964)*** Most schedules in use in the world today call for two doses 1 to 2 months apart followed by a third dose 6 to 12 months later. After two doses 4 weeks or more apart, almost all adults produce antitoxin levels higher than 0.01 IU/mL.371 Other workers have indicated a weaker immune response in elderly individuals ***(Kishimoto,***

*S., 1980; Simonsen, O.,1987; Murphy, S.M., 1995)* Nevertheless, in most adults, routine boosters every 10 years are sufficient to maintain immunity. *(McCarroll, J.R., 1962; Gottlieb, S., 1964; Simonsen, O., 1987)*

**2.2.6.8 Administration to Women of Child-Bearing Age for Prevention of Neonatal Tetanus**

A minimum of two doses of tetanus toxoid at least 1month apart with the last dose at least 2 weeks before the estimated date of delivery appears to provide protective levels of antibody for well above 80% of newborns. *(Galazka, A.M., 1993)* WHO has adopted a schedule of five tetanus toxoid doses administered during a minimum of 2.6 years and preferably more than 10 years to induce sustained levels of circulating antitoxin in all vaccinated women for the duration of their reproductive years. *(W.H.O., 1986)* (Table 27-3).The EPI, established in 1974 to provide multiple vaccines to infants, also included the immunization of women in pregnancy to prevent tetanus neonatorum.

**2.2.6.9 Duration of Immunity and Booster Immunization**

After each subsequent tetanus toxoid injection, antitoxin levels peak within 2 weeks, fall rapidly during 2 months, and then fall more gradually in the years following *(Evans, D.G., 1943)*. Tetanus toxoid prophylaxis in wound management is recommended.

The consequences to population immunity of the lack of compliance with the booster policy are evident in the results of the recent population-based tetanus antitoxin serosurvey in the United States: The proportion of the population with protective levels of antitoxin declines with increasing age, leaving 20% of those 40 to 49 years of age unprotected and 70% unprotected by the age of 70 years;

**2.2.6.10 Booster Immunization for Prevention of Maternal and Neonatal Tetanus**

The WHO recommendation for tetanus immunization of women of child-bearing age in countries with and without well-established routine childhood vaccination is shown in Table 27-3. *(W.H.O., 1986; W.H.O., 1999)*

**2.2.6.11 Immunization in Immunodeficiency**

Because tetanus toxoid-containing vaccines (DTP, DtaP, DT, Td, and TT) are inactivated, they are safe for use in immunocompromised individuals, including those with congenital immunodeficiencies, human immunodeficiency virus (HIV) infection, hematologic or other malignancies, stem cell transplants (SCTs) or organ transplants,

and chronic renal failure (CRF). Most of these conditions are associated with reduced responsiveness to tetanus toxoid vaccination or shorter duration of protection. Anti-tetanus antibody determinations may be indicated in immunocompromised individuals to assess responses to vaccination and duration of protection, and to guide the use of supplemental tetanus toxoid doses. Adults and infants infected with HIV generally respond to tetanus toxoid vaccination with increases in serum antitoxin levels. ***(Moss, W.J., 2003)***

**2.2.6.12 Adverse Events Following Immunization**

Whereas mild local reactions are relatively common after receipt of tetanus toxoid, more serious reactions, including neurologic and hypersensitivity reactions, are less. The rates and severity of adverse events in recipients of tetanus toxoid are influenced by the number of prior doses and level of pre-existing antitoxin, perhaps the amount of toxoid in the dose, the type and quantity of adjuvant, the route of injection, the presence of other antigens in the preparation, and perhaps the presence of organomercurials used as preservative.

The most common adverse event following injection of tetanus toxoid is a local reaction (0% to 95% of recipients). ***(Myers, M.G., 1982)*** Among recipients of a booster dose of adsorbed toxoid, 50% to 85% experience pain or tenderness at the injection site and 25% to 30% experience edema and erythema.427 More severe local reactions characterized by marked swelling occur in fewer than 2 % of vaccinees.

Typically, these reactions began within 2 to 8 hours after toxoid administration in the deltoid. ***(McComb, J.A., 1961; Eisen, A.H., 1963; Levine, L., 1981)*** Preformed antibody apparently forms complexes with the deposited toxoid to induce an inflammatory response (Arthus reaction, or type III hypersensitivity). ***(Eisen, A.H., 1963; Levine, L., 1981)*** Aluminum adjuvant in adsorbed products theoretically can invoke local inflammatory responses more frequently than fluid toxoid because the presence of adjuvant can induce activation of complement and stimulate macrophages. ***(Collier, I.H., 1979; Edelman, R., 1980)***

The frequency of local reactions is increased when toxoid is administered subcutaneously rather than intramuscularly. This is particularly true for adsorbed toxoid ***(E.P.I., 1982)***; aluminum adjuvant can cause sterile abscesses when given subcutaneously. ***(Edelman, R., 1980)*** Use of jet injectors, which deposit some toxoid in

the subcutaneous tissue, results in twofold higher rates of edema at the administration site than intramuscular injection by needle.

Fever can accompany a local response. Other systemic symptoms such as headache or malaise are reported less frequently than fever. Lymphadenopathy also can occur after toxoid inoculation. *(Eisen, A.H., 1963)*

Peripheral neuropathy, particularly brachial plexus neuropathy, has been reported to occur hours to weeks after tetanus toxoid administration. *(Quast, U., 1979)*

Tetanus toxoid occasionally induces an immunoglobulin E (IgE) response, particularly with aluminum salt-adsorbed toxoid *(Vassilev, T.L., 1978; Nagel, J., 1977; Matuhasi, T., 1982)* True anaphylactic (type I hypersensitivity) reactions to purified tetanus toxoid are rare *(Inst Med Vac Safety Comm, 1994)*.

**2.2.6.13 Simultaneous Administration with Other Vaccines**

Tetanus toxoid has been combined with diphtheria toxoid, pertussis vaccines (both whole cell and acellular), and Hib conjugate vaccines without compromising the immune response or substantially enhancing adverse events associated with the tetanus component.

**2.2.7. Future Prospects**

**2.2.7.1 Oral vaccines**

Attempts have been made to administer tetanus toxoid orally after enclosing it in an acid resistant granule to facilitate mass immunization and greater acceptability. A 10-50 times greater dose of toxoid is required to generate immune response, if given orally. A synthetic vaccine which is much more potent may-be the future candidate for oral vaccine against tetanus. Development of a synthetic oligopeptide simulating sequential antigenic determinants of toxin molecule is on the card. It is believed that such oligopeptides have better capability to penetrate intestinal barriers. *(Singh, H., 1993)*

**2.2.7.2 Subunit vaccines**

Enormous research has been made onto the structure and functions of different parts of tetanus toxin. Two subchains can be separated and each has been shown to induce antibodies against other in experimental animals. However, the subchains, even after separation, exhibit residual toxicity, which can be abolished by the available immunological techniques. A procedure for the purification of tetanus toxin on a column

of immobilised gangliosides has been standardised and may yield large quantity of toxin subchains.

Fragment C can be obtained with papain digestion. This fragment is nontoxic but gives rise to neutralising antibodies. However, the cost of separation and purification of fragment C is prohibitive.

**2.2.7.3 Recombinant DNA vaccine against tetanus**

Fragment C of tetanus toxin has been expressed in an insoluble form in *E.coli* and partially purified. In experimental animals such as mice, protective immunity could be induced with this. Although this insoluble product cannot be used in human beings, it may facilitate the production of conventional vaccines.

**2.2.7.4 Tetanus vaccine as carrier of other vaccines**

Most of the capsular polysaccharides are unable to stimulate T cells of the immune system because of the repetitive nature of their structure. These stimulate good immune response in adults but fail to do so in children less than 2 years of age. This problem can be overcome by artificially conjugating the polysaccharide with tetanus toxoid (or diphtheria toxoid) so as to add T-cell epitopes for better immune response even in children. Pneumococci, meningococi and Haemophilus influenzae type b are some of the potential candidates, which can be linked up with tetanus toxoid to improve upon the immune response. Similarly beta subunit of human chorionic gonadotrophin (beta-HCG) has been coupled with tetanus toxoid in the antifertility vaccine currently being vigorously pursued. ***(Singh, H., 1993)***

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## 2.3 DIPHTHERIA

Diphtheria has various distinctions to its credit in the studies on microbial diseases. Various achievements and discoveries were made for the first time for this disease and subsequently replicated for other bacterial disorders. It was the first infectious disease to be studied fully- its clinical and bacteriological identification, its cure by a specific antiserum and its prevention by a specific vaccine.

### 2.3.1. History of the disease and vaccine

Diphtheria is an ancient disease, which finds mention in records pertaining to times of Hippocrates. Epidemics of this disease were common and responsible for high mortality. The disease had been known in different parts of world as palatal paralysis, garrotillo (because of death from suffocation), throat distemper and so on. The name diphtheria was assigned by Bretonneau in 1826. It is a derivative of Greek word *diphterie,* which denotes a prepared skin resembling the leathery appearance of diphtheritic membrane. (***English, P.C., 1985***)

Diphtheria is an acute communicable upper respiratory illness caused by *Corynebacterium diphtheriae,* a gram-positive bacillus. 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. Extensive membranes and organ damage are caused by local and systemic action of a potent exotoxin produced by some strains of *C. diphtheriae.* (***Burrows, W., 1959; Singh, H., 1993***)

- 1883: Klebs first described the characteristic organisms in stained preparations of diphtheritic membranes
- 1884: Loeffler reported the successful growth of these organisms in culture
- 1888: Roux and Yersin found that sterile broth filtrates of cultures of the organism, when injected, into animals produced death thus demonstrating the presence of a potent exotoxin.
- 1901: Noble prize to von Behring for preparation of *antitoxin* which protected nonimmune animals when challenged by virulent organisms
- 1907: Theobald Smith who noted that long-lasting immunity to diphtheria could be produced in guinea pigs by the injection of mixtures of diphtheria toxin and antitoxin and suggested use in humans.

- 1913: Schick introduced a skin test for immunity that consists of the injection of a small, measured amount of diphtheria toxin; in immune persons, circulating antibody neutralizes the toxin, and no local lesion is observed.
- 1920: Ramon showed that diphtheria toxin, when treated with heat and formalin, lost its toxic properties but retained its ability to produce serologic protection against the disease.
- 1926: Glenny and co-workers found that alum-precipitated toxoid was more immunogenic.
- 1940: Diphtheria toxoid was combined with tetanus toxoid and pertussis vaccine as diphtheria and tetanus toxoids and whole-cell pertussis vaccine (DTP).
- 1974: Expanded Program of Immunization launched by WHO

**2.3.2. Clinical Description**

Classical diphtheria has an insidious onset after an incubation period of 1 to 5 days (rarely longer). Symptoms of diphtheria are initially nonspecific and mild; throughout the course of the disease, fever does not usually exceed 38.5°C (101.3°F). Other early symptoms in children include diminished activity and some irritability. About a day after onset, small patches of exudate appear in the pharynx. Within 2 or 3 days, the patches of exudate spread and become confluent and may form a membrane that covers the entire pharynx, including the tonsillar areas, soft palate, and uvula. This membrane becomes grayish, thick, and firmly adherent. Efforts to dislodge the membrane result in bleeding. Anterior cervical lymph nodes become markedly enlarged and tender. The lymph node swelling is associated with considerable inflammation and edema of the surrounding soft tissues, giving rise to the so-called bull neck appearance, which is associated with a higher morbidity and mortality. In untreated patients, the membrane begins to soften about a week after onset and gradually sloughs off, usually in pieces but sometimes as a single unit. As the membrane detaches, acute systemic symptoms, such as fever, begin to disappear. ***(Burrows, W., 1959)***

Although pharyngeal diphtheria is by far the most common form of disease seen in unimmunized populations, other skin or mucosal sites may be involved. Laryngeal diphtheria (25%, mostly in children less than 4 years of age), isolated nasal diphtheria is uncommon (about 2% of cases), Cutaneous, aural, vaginal, and conjunctival diphtheria together account for about 2% of cases. ***(Singh, H., 1993)***

### 2.3.2.1 Complications

The major threat from laryngeal diphtheria is respiratory obstruction (croup). Because of edema of the upper respiratory tract, pharyngeal and nasal diphtheria are frequently associated with secondary otitis media and sinusitis. The majority of deaths from diphtheria are due to the effects of absorbed diphtheria toxin on various organs; severe complications from toxin absorption include acute systemic toxicity, myocarditis, and neurologic complications, primarily peripheral neuritis.Neurologic complications of diphtheria are primarily toxic peripheral neuropathies and occur in approximately 15% to 20% of cases. In severe cases, palatal paralysis with consequent nasal voice and nasal regurgitation of ingested fluids may occur during the acute membranous phase, particularly with extensive pharyngeal disease, and are believed to be attributable to local effects of the toxin. Diphtheric membrane is shown in **Photo 4**

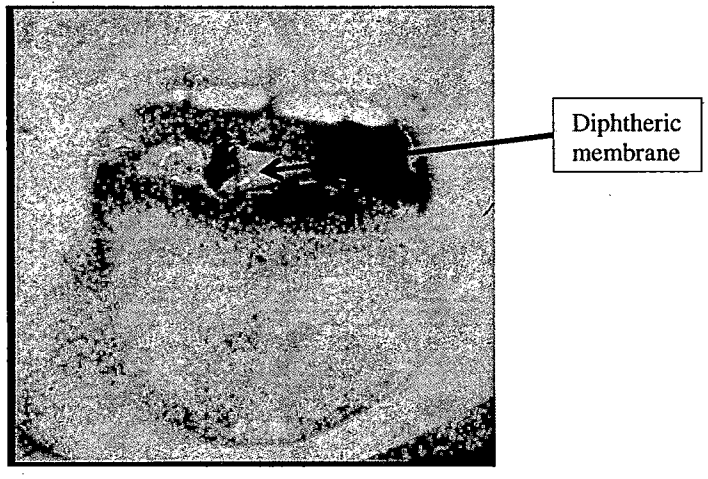



**Photo 4: Diphtheric membrane**

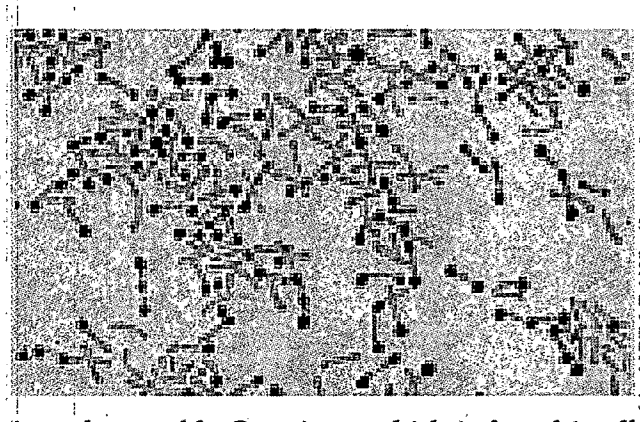
### 2.3.3. Biology of the Organism and Pathogenesis of Infection

Corynebacterium diphtheriae is a pleomorphic organism. Maximum pleomorphism is seen when the bacterium is grown on nutritionally inadequate medium and repeatedly subcultured. The bacterium measures 3-6 μm x 0.6-0.8 μm, is slender and sometimes has swollen ends. Most organisms of this species contain 2-3 granules at the swollen ends, which give reddish purple colour when stained with Loeffler's alkaline methylene blue. Rest of the bacterium is unevenly stained with this dye. The granules because of their colour reaction are also known as metachromatic granules. Other common names for these are Babes Ernst's granules and volutin granules. It is believed that these granules

store energy sources for the bacterium. Their presence is also associated with toxigenicity of the bacterium, though toxigenic strains without any granules have also been frequently encountered. C.diphtheriae is gram positive and very easy to decolourize. It can be stained with simple stains such as methylene blue. It is non-acid-fast. Granules can be better visualised with Albert's stain which stains the granules as bluish black and the cytoplasm as green. These bacilli exhibit characteristic arrangement in smear preparations. Adjacent bacteria lie at various angles to each other giving V or L appearances which collectively resemble arrangement of Chinese letters. This unusual arangement is because of incomplete separation of daughter cells at the moment of division. This bacterium does not possess flagellae but fimbriae are formed by many C.diphtheriae. The bacterium may also branch occasionally but mycelia are not formed. ***(Burrows, W., 1959; Singh, H., 1993).***

*Corynebacterium diphtheriae* is a slender gram-positive bacillus, usually with one end being wider, thus giving the often described club-shaped appearance. On smear, the organisms often have a "pick-up sticks" relationship, assuming parallel (palisade-like), V- or L-type patterns.**(Figure 14)**The organisms are resistant to environmental changes, such as freezing and drying. There are four biotypes of *C. diphtheriae- gravis, mitis, belfanti, and intermedius.* (***Funke, G., 1997***) No consistent differences are found in severity of disease caused by different biotypes.

**Figure 14: *Corynebactrium diphtheriae* bacterial colony**



The cell wall contains a heat-stable O antigen, which is found in all *corynebacteria.* The cell wall also contains K antigens, which are heat-labile proteins that differ among strains of *C. diphtheriae.* The K antigens play two roles in relation to humans: first, they appear to be important in the establishment of infection; and second, they produce local type-specific immunity. Cord factor, which is a toxic glycolipid, which has been shown to

disrupt mitochondria, depress cell respiration, and interfere with oxidative phosphorylation. (*Kato, M., 1970*)

### 2.3.4. Diphtheria Toxin

The exotoxin produced by *C. diphtheriae* is by far the most important pathogenetic factor. The ability to produce toxin in strains of *C. diphtheriae* results from a nonlytic infection by one of a series of related bacteriophages containing a genetic sequence encoding the toxin. The phage integrates into specific sites present in *C. diphtheriae* and other *Corynebacterium* species. The presence of the phage is thought to confer a survival advantage to the bacterium by increasing the probability of transmission in a susceptible population; transmission may be facilitated by local tissue damage resulting from the toxin. (*Pappenheimer, A.M., 1973; Collier, R.J., 1975*) The sequence of diphtheria toxin has been demonstrated to be highly conserved in *C. diphtheriae* strains, suggesting that immunologically important differences among the toxins produced by different strains are unlikely to occur. Once integrated, the tox gene is part of a multiple bacterial gene operon; other bacterial gene products in this operon are involved in the liberation and uptake of host iron. The entire operon is under the control of a repressor gene, dtxR, which in the presence of iron binds to and inhibits the tox gene; toxin is produced only under low iron conditions. The optimum level of iron for toxin production is 0.1 mg/litre. The formation of toxin is inhibited if the iron concentration is elevated to 0.5 mg/litre. (*Tao, X., 1994*)

Diphtheria toxin is a polypeptide with a molecular weight of about 58,000. The toxin is secreted as a proenzyme, requiring enzymatic cleavage into two fragments (fragments A and B) to become active. **(Figure 15 and 16).** Fragment B is responsible for attachment to and penetration of the host cell. Although nontoxic by itself, fragment B appears to be the antigen responsible for clinical immunity. The receptor domain of fragment B binds to a cell surface receptor, CD9 as a co-receptor. *(Cha, J-H., 2000)*After receptor-mediated endocytosis and penetration of the cell, fragments A and B are detached. The released fragment A is the toxic moiety and acts by inhibiting protein synthesis, resulting in cell death. (*Collier, R.J., 1975*) Unless cell penetration occurs, fragment A is inactive. (*Vaughan, T.J., 1992*)

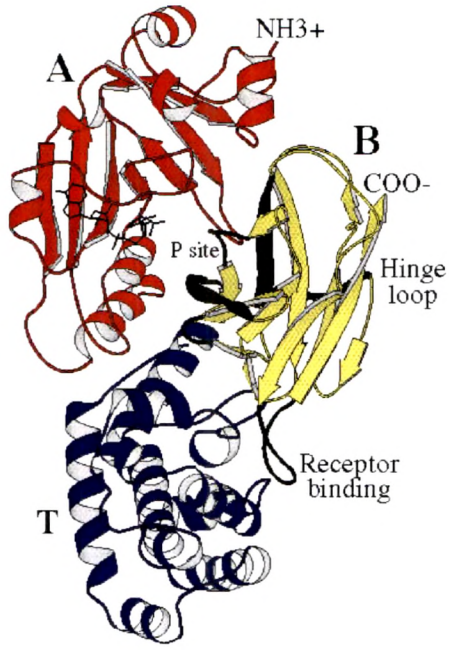



**Figure 15: The Diphtheria Toxin (DT) Monomer-**

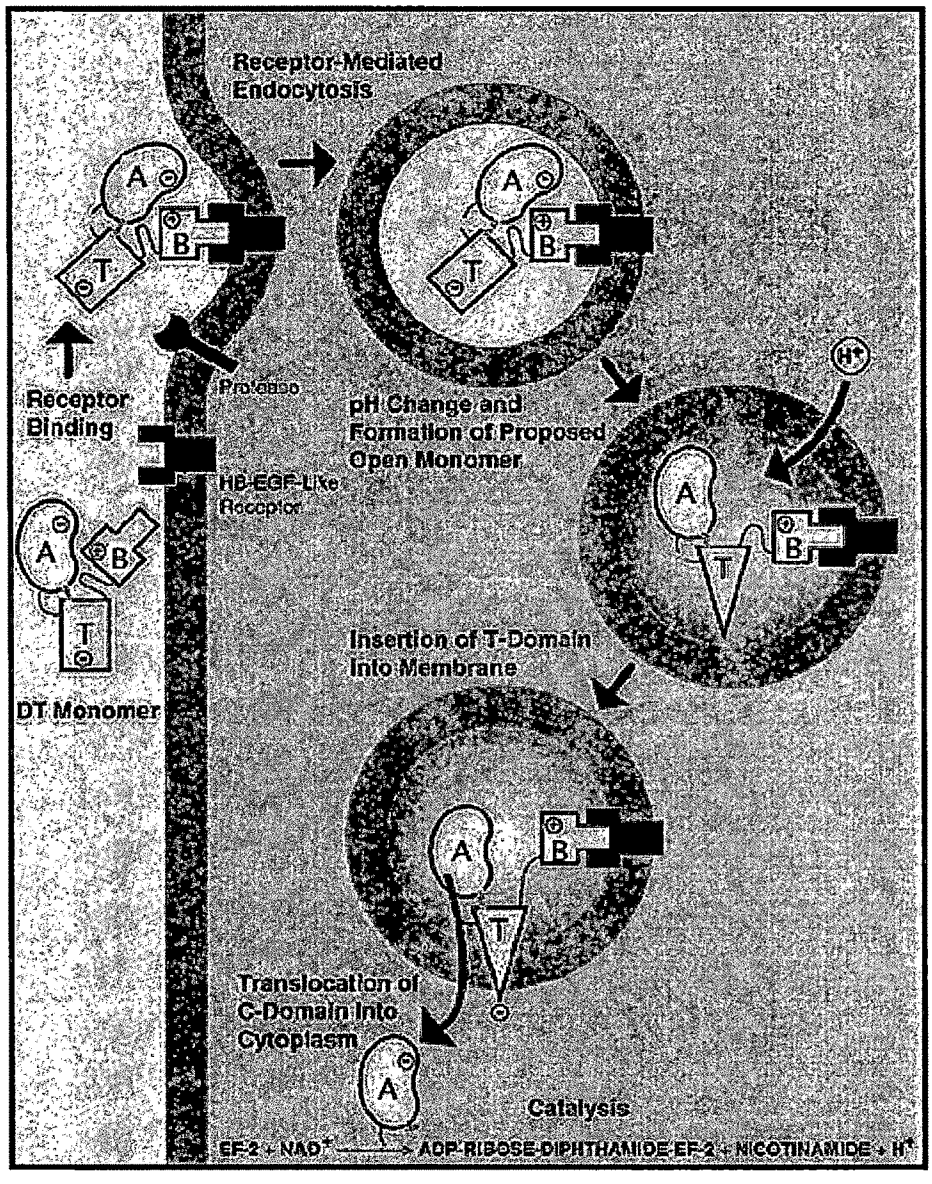
**A (red) is the catalytic domain; B (yellow) is the binding domain which displays the receptor for cell attachment; T (blue) is the hydrophobic domain responsible for insertion into the endosome membrane to secure the release of A. The protein is illustrated in its "closed" configuration.**

The diphtheria toxin can be converted into toxoid wherein it retains its immunogenicity but loses the virulence by prolonged storage, incubating it at a temperature of 37°C for 4.6 weeks or by subjecting it to the action of formaldehyde or acidic pH. Many reagents have been used to modify the toxicity of diphtheria toxin without a parallel loss of antigenicity.

Almost any reagent that will react with free α-amino groups of lysine in purified toxin will greatly reduce its toxicity while keeping its antigenicity intact. In addition to formaldehyde these reagents include ketene, β – propiolactone, dinitrofluorobenzene and glutaraldehyde. The ideal toxoid reagent is the one that not only brings about complete detoxification but also actually enhances antigenicity by cross-linking A and B fragments to one another and by producing crosslinked dimers and polymers. The formol-toxoid is more heterogenous than toxin, far more stable to denaturation by heat

or by acid and is more resistant to the action of proteolytic enzymes. It has no enzymatic activity and does not react with toxin-specific receptors on the surface of the cells. *(Rappuoli R., 1997).*

**Figure 16: Uptake and activity of the diphtheria toxin in Eukaryotic cells**




**The figure above was redrawn from the Diphtheria Toxin Homepage at UCLA. A represents the A/B toxin's A (catalytic) domain; B is the B (receptor) domain: T is the hydrophobic domain that inserts into the cell membrane.**

On mucous membranes, the toxin causes local cellular destruction, and the accumulated debris and fibrin result in the characteristic membrane. More important, absorbed toxin is responsible for remote manifestations affecting various organs, including the myocardium, nervous system, kidneys, and others. Because the lethality of diphtheria is almost entirely determined by the organism's toxin, clinical immunity depends primarily on the presence of antibodies to the toxin. *(Rappuoli R., 1997)*

### 2.3.5 Protective Levels of Antitoxin

Several lines of evidence suggest that persons with diphtheria antitoxin levels of less than 0.01 IU/mL should be considered susceptible. Clinical diphtheria was rare among individuals with a negative Schick test; the minimal serum antitoxin level associated with a negative Schick test was approximately 0.005 IU/mL. An antitoxin level of 0.01 IU/mL is the lowest level giving some degree of protection, and 0.1 IU/mL is considered a protective level of circulating antitoxin. Levels of 1.0 IU/mL and above are associated with long-term protection. *(Efstratiou, A., 1994)*

### 2.3.6 Epidemiology

Active immunization of children with diphtheria toxoid has markedly altered the epidemiology of diphtheria, reducing diphtheria to extremely low levels in both developed countries and many developing countries. However, diphtheria continues to produce substantial childhood morbidity and mortality in developing countries with incompletely implemented childhood immunization programs. *(Galazka, A.M., 1975)*

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 of 0.01 IU/ml. **(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)**. 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 **(Relyveld, E., 1997)**.

Humans are the only natural host for *C. diphtheriae*. Transmission is person to person, most likely by intimate respiratory and physical contact. Clinical case as well as asymptomatic carrier can shed bacteria in droplets, pharyngeal secretions, skin exudates and aural discharges. In classical diphtheria the site of infection is nasopharynx. The bacilli multiply here and produce exotoxin. The toxin causes local necrosis and bacterial multiplication and toxin production continues in debris. Cutaneous lesions appear to be important in transmission in warm climates or under conditions of poor hygiene. In temperate climates, diphtheria occurs year-round but most often during colder months, probably because of the close contact of children indoors. Preschool and school-age children are most often affected by respiratory diphtheria. Transplacental antitoxic immunity to diphtheria is present at birth in most infants but declines to nonprotective levels during the second 6 months of life. Thereafter, the proportion of immune children (Schick negative) in unimmunized populations gradually increases to 75% or more, presumably owing to subclinical infection with the organism, perhaps repeated infection. (***Burnet, M., 1972***)

For the United States before 1900, during 1860 to 1897, death rates from diphtheria ranged between 46 and 196 per 100,000 population annually, By 1900, a considerable fall in death rates had occurred, and the death rate continued to decline from 40 to 15 per 100,000 for the next 20 years, presumably owing to the therapeutic use of diphtheria antitoxin

In contrast, national diphtheria immunization campaigns did not take place in Britain until the early 1940s. During World War I, a major outbreak spread throughout western Europe; well above 1 million cases were reported.(***Stowman, K.,1947; Stuart, G., 1945***) During World War II, outbreaks linked to Europe occurred in North America as well.

By the late 1950s, diphtheria was markedly reduced in the United States, but disease continued to occur in some geographic areas. Improved childhood immunization in Mexico and other developing countries as part of the Expanded Programme on Immunization (EPI) beginning in the late 1970s is likely to have contributed to improved diphtheria control in the United States by reducing importations of toxigenic strains.

Although diphtheria has become a rare disease in most developed countries, a major epidemic of diphtheria began in the Russian Federation in 1990 and subsequently spread

throughout the countries of the former Soviet Union (Fig. 13-2), with more than 157,000 cases and 5000 deaths reported between 1990 and 1998. ***(Dittmann, S., 2000)***

In developing countries, the implementation of the EPI program has led to dramatic falls in the global number of reported cases of diphtheria since 1980.

### 2.3.7 Treatment of Diphtheria

Many studies have demonstrated the efficacy of therapy with antitoxin in reducing mortality from diphtheria primarily by preventing cardiovascular toxicity. ***(English, P.C., 1985)*** Only 4.8% of 1168 patients developed paralysis when antitoxin was administered no later than the second day of illness, in contrast to 12.1 % of 1375 patients who received antitoxin on the fourth day of the disease or later. Antitoxin is given intramuscularly or intravenously ***(Tasman, A., 1958).*** The entire therapeutic dose should be administered at one time, and the amount of antitoxin recommended varies between 20,000 and 120,000 units. The longer the interval since onset, the higher should be the dose of antitoxin. Unfortunately, toxin that has already entered host cells is not affected by antitoxin. Penicillin or erythromycin should be given to hasten clearance of the organism ***(Farizo, K.M., 1993).*** Treatment should be continued until at least two consecutive daily cultures fail to demonstrate *C. diphtheriae.*

#### 2.3.7.1 Active Immunization

The combination preparation, toxin-antitoxin, was rapidly accepted as an active immunizing agent. It was widely used in the United States beginning in 1914 and was found to protect approximately 85% of recipients. The Schick test consists of the intradermal injection of a minute amount of diphtheria toxin. The test result is ordinarily read after 48 hours; erythema and induration of 1 cm or more indicates susceptibility to diphtheria. Because the correlation between results of the Schick test and clinical immunity to diphtheria is reasonable, although not perfect, the test served for many years as an acceptable surrogate for clinical immunity to diphtheria.

The immunogenicity of diphtheria toxoid, as well as that of tetanus ooxoid, is enhanced by the adjuvant effects of both pertussis vaccine and the aluminum salt. ***(Greenberg, L., 1948; Spiller, V., 1955)*** In recent years, diphtheria and tetanus toxoids with acellular pertussis components (DTaP) have been licensed, and various other combinations of

DTaP with Hib vaccine, inactivated poliovirus vaccine, and hepatitis B vaccine have been developed.

#### 2.3.7.2 Passive Immunization

Equine diphtheria antitoxin is prepared by hyperimmunizing horses with diphtheria toxoid and toxin. *(U.S.FDA, 1985)* There must be at least 500 units of antitoxin per milliliter, and sterility is attained by microfiltration. No antiserum or hyperimmune globulin of human origin is currently available.

#### 2.3.7.3 Postexposure Use of Antitoxin and Toxoid

Antitoxin is not recommended for exposed, susceptible persons, particularly in view of the high rates of subsequent serum sickness and occasional anaphylaxis. The preferred treatment for exposed, unimmunized, asymptomatic persons is to obtain a throat culture, begin immunization with a preparation containing diphtheria toxoid that is appropriate for age, and institute prophylaxis with erythromycin or penicillin for 7 days, during which time the patient must be kept under surveillance. *(Farizo, K.M.,1993)*

### 2.3.8. Current Production

The Park Williams 8 (PW8) strain of *Corynebacterium diphtheriae* (isolated in 1896 from the throat of a case with mild diphtheria) has been shown to be satisfactory for producing potent diphtheria vaccine. It gives greatest possible quantity of toxin during the growth phase of the microorganism and ever since its use the amino acid sequence of the tox gene responsible for toxin production has been conserved. Before the commencement of production, the strain (PW8) is identified by a record of its history and all tests are made to verify strain characters. The strain is maintained as a freeze-dried culture. From this, the working seed lot is prepared.

The PW8 strain, unlike typical C.diphtheriae utilizes flavoprotein as a terminal oxidase rather than cytochromes a+a3. Hence the rate of bacterial oxygen consumption is directly proportional to its partial pressure even up to atmospheric pressure and above. In order to ensure an adequate supply of oxygen, commercial toxin fermentors are used into which air under pressure or oxygen is sparged with vigorous stirring. In the presence of

adequate nutrition and optimum temperature of 34-35°C, the final growth attained may reach 20-30 gram dry weight of bacteria per litre.

Although several semisynthetic media for industrial growth of diphtheria bacilli have been described, most manufacturers prefer to grow these in enzymic digests of beef. Precautions must be taken to see that digestion has proceeded sufficiently. The supply of glucose and pH control are two critical factors in obtaining good yield of toxin. If glucose is present in excess or if oxygen should become limiting, oxidation is incomplete and acetic, propionic and lactic acids accumulate and bring down the pH of the medium. The moment pH falls down to below 6.0-6.5, no toxin is produced. Glucose-free maltose has been considered superior to glucose as the source of energy because the enzyme maltase produced by PW8 has a weak affinity for maltose and hence glucose is liberated slowly and is the growth-limiting factor. Under these conditions the PW8 strain is grown in fermentors for 36-48 hours until the concentration of toxin in supernatant reaches 150 to 250 Lf/ml (1 Lf = 2.5 μg of diphtheria toxin). When the fermentation is complete, the bacteria are removed by centrifugation and formaldehyde is added to the supernatant to a final concentration of 0.75 %. The supernatant is then stored at 37°C for four to six weeks to allow complete detoxification of diphtheria toxin. Under ideal conditions, diphtheria toxin may constitute 5-10% of all proteins synthesized by PW8 strain and 75% of all extracellular proteins secreted. Hence the isolation of toxic proteins in a relatively high state of purity and in good yield is relatively easy. Purification may either precede or follow detoxification. Several methods have been described for the purification of diphtheria toxin. Typically, the toxin in the culture supernatant can be concentrated through diafiltration, precipitated by ammonium sulphate and, if necessary, further purified by gel filtration or ion-exchange chromatography. With these methods purity to the extent of 90% can be obtained. The partially purified or highly purified toxin preparations can then be detoxified by the conventional formaldehyde treatment.

Early studies carried out with purified diphtheria toxin showed that toxoids prepared from highly purified toxin became toxic after dilution and removal of formaldehyde. Addition of lysine to detoxification solution results into formation of stable and immunogenic toxoids. Glutaraldehyde, along with lysine, is used in France to detoxify diphtheria toxin and it results into formation of polymerized antigen (POLAN) and this

has been successfully used in immunizing infants and children in France without any noticeable adverse reactions.

The bulk purified toxoid is tested for the presence of diphtheria toxin. The method essentially is same as described elsewhere in this Chapter. The amount of toxoid to be injected is 1 ml having at least 500 Lf of toxoid. The bulk purified toxoid passes the test if no guinea pig shows symptoms of specific intoxication within six weeks of injection and if at least 80% of the animals survive test period. Each bulk purified toxoid is also tested to ensure that reversion to toxicity does not take place on storage. The bulk purified toxoid is diluted in order to obtain the same concentration and chemical environment as is present in the final bulk vaccine except the adjuvant. Diluted toxoids that have been stored for six weeks at 37°C are tested. Intradermal test in guinea pigs is considered suitable. The antigenic concentration in Lf units of final purified toxoid bulk is ascertained and it passes the test if it contains no fewer than 1500 Lf per mg of protein (non-dialyzable) nitrogen. From the final purified toxoid bulk, final bulk is prepared which should contain not more than 30Lf of toxoid per single human dose (0.5 ml), preservative (other than phenol) can be added if vaccine is to be dispensed in multidose containers, and an adjuvant (aluminum or calcium compounds) is generally used as a mineral carrier with concentration of aluminium not exceeding 1.25 mg and that of calcium 1.3 mg per single human dose. The bacterial and mycotic sterility are tested along with specific toxicity in guinea pigs as discussed earlier. The potency of diphtheria vaccine used for immunization of children shall not be less than 30 IU per single human dose. The formaldehyde residual content in final bulk is not more than 0.2 gram/litre and the pH is between 6.0-7.0. The diphtheria toxoid should not be frozen and storage at 2-8°C has been found to be satisfactory.

### 2.3.9. Available preparations and Immunization schedule

For routine immunization of children, five doses are recommended (at 2, 4, 6, and 15 to 18 months and at school entry before the seventh birthday). The fourth dose should be administered at least 6 months after the third dose. Follow-up of a small group of children born at less than 29 weeks' gestation suggests lower diphtheria antibody levels at age 7, following a five-dose series, compared to children born at term. Diphtheria and Tetanus Toxoids, Adsorbed, for Pediatric Use (DT) is recommended for the primary immunization of children younger than 7 years in whom pertussis vaccine is

contraindicated. DT contains 10 to 12 Lf of diphtheria toxoid; infants who begin the series before 1 year of age should receive DT at 2, 4, 6, and 15 to 18 months of age. For unimmunized children 1 to 7 years of age, two doses 2 months apart and a third dose 6 to 12 months later constitute primary immunization. *(CDCP, 1991)*

Tetanus and Diphtheria Toxoids, Adsorbed, for Adult Use (Td) contains approximately the same amount of tetanus toxoid as do DTP and DT, but the amount of diphtheria toxoid is reduced to no more than 2 Lf/dose. This reduction minimizes reactivity in persons who may have been sensitized previously to diphtheria toxoid and is sufficient to provoke satisfactory anamnestic responses in previously immunized persons. In addition, in previously unimmunized older children and adults, Td is satisfactory for primary immunization *(Myers, M.G., 1982)* when administered as a three-dose series, with the second dose given 4 to 8 weeks after the first dose and the third dose 6 to 12 months after the second dose. *(CDCP,1991)* Td should be administered approximately every 10 years after the completion of childhood immunization. Although Td is slightly more reactive than tetanus toxoid alone, it is preferable to monovalent tetanus toxoid for prophylaxis of tetanus after wounds to maintain satisfactory population immunity against diphtheria. DTaP may be administered simultaneously with hepatitis B vaccine, Hib vaccine, inactivated poliovirus vaccine, and pneumococcal conjugate vaccine to infants at ages 2, 4, or 6 months.

Preparations containing diphtheria toxoid should always be injected intramuscularly, not subcutaneously. According to the recommended schedule for the World Health Organization's EPI, DTP is administered at 6, 10, and 14 weeks without additional doses.

**2.3.10. Results of Immunization**

No controlled clinical trial of the efficacy of the toxoid in preventing diphtheria has ever been conducted for three reasons. First, given the serious nature of the disease and the clear perception of benefit first from the toxin-antitoxin combination and subsequently from the toxoid, its value seemed obvious to early investigators. Second, the development of surrogate approaches to assessing immunity (the Schick test and serologic methods) made such trials unnecessary. Third, the early appearance of strong presumptive evidence that the toxin-antitoxin preparation and the toxoid were effective made such trials unethical.

There is complete disappearance of the disease in countries in which immunization has been widely employed. During outbreaks of diphtheria, rates of disease are negligible among immunized persons. When partially or, rarely, fully immunized individuals acquire diphtheria, the disease is milder and complications are fewer. A good correlation has been established between clinical protection and the presence of serum antibody to the toxin, whether resulting from disease or from immunization.

After three doses of diphtheria toxoid, virtually all infants develop diphtheria titers greater than 0.01 IU/mL. (***Orenstein, W.A.,1983***) High maternal antibody titers suppress, but do not prevent, adequate responses of infants to two doses of vaccine; and after the third dose the suppressive effect is gone. When the toxoid is used for primary immunization of adults, data suggest that virtually all adults develop diphtheria antitoxin titers greater than 0.01 IU/mL after administration of three doses of diphtheria toxoid and that most develop titers greater than 0.1 IU/mL. ***(Myers, M.G., 1982)***

Before the World Health Organization's EPI began, it was estimated that close to a million cases of diphtheria occurred annually in the Third World, with 50,000 to 60,000 deaths. ***(Walsh, J.A., 1979)*** From 1980 to 2000, reported cases of diphtheria globally decreased from 97,811 to 9593; with control of the outbreak in the former Soviet Union, almost 80% of cases worldwide in 2000 were reported from the African and Southeast Asian regions. **Figure 17 and 18** shows global annual reported incidence for diphtheria and DTP3 coverage for the period of 1980-2003 and only 2003 respectively. WHO DTP3 coverage is up to 90% and number are cases are bellow 10,000. ***(www.who.int/vaccines-surveillance)***

**Figure 17: Diphtheria Global annual reported incidence and DTP3 coverage (1980-2003)**

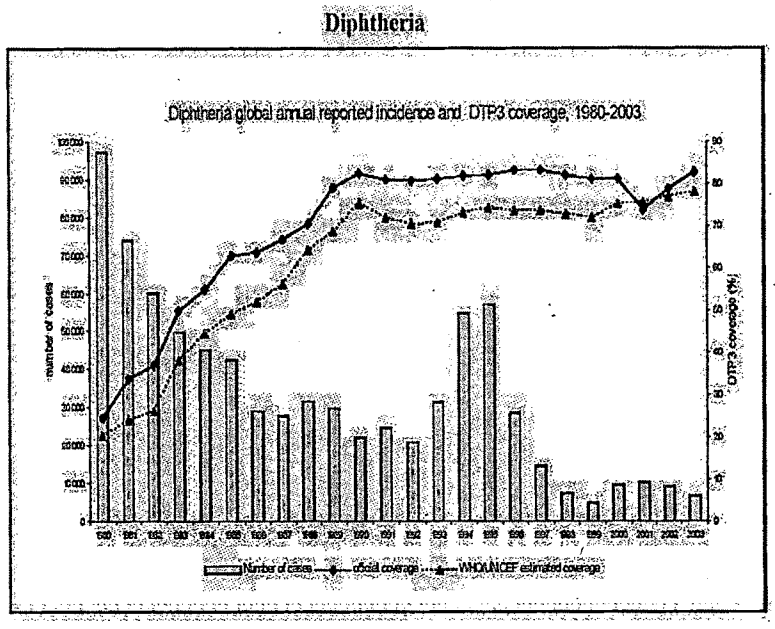
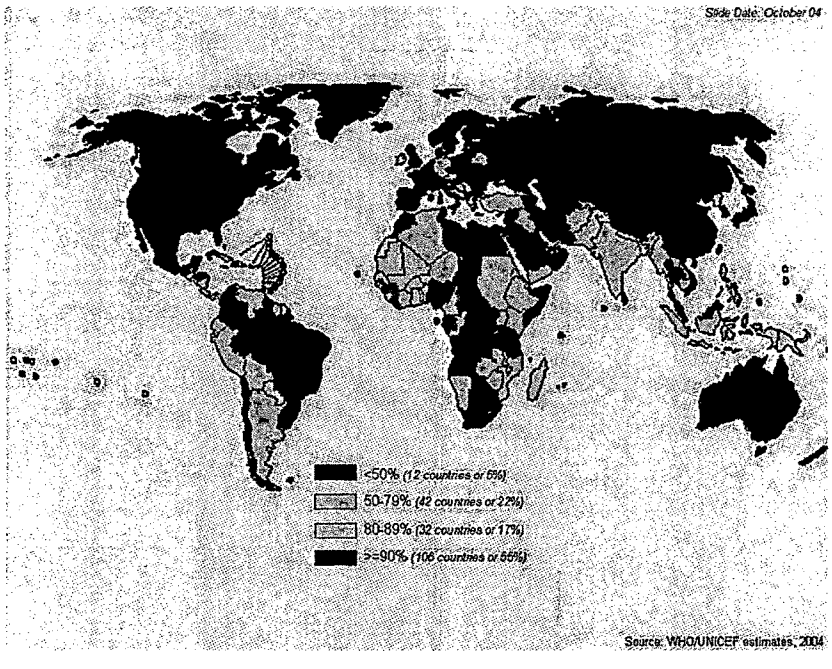



**Figure 18: Immunization coverage with DTP3 vaccines in Infants, 2003**



### 2.3.11. Duration of Immunity

A serologic study in England and Wales showed that only 29% of adults age 60 years and older had diphtheria antitoxin titers of 0.01 IU/mL or greater. (*Maple, P.A.C., 2001*) A similar pattern of susceptibility is seen among elderly persons in other countries of Western Europe. It has been thought that 70% or more of a childhood population must be immune to diphtheria to prevent major community outbreaks; the herd immunity threshold has been estimated at 80% to 85%, based on the average age of infection in the prevaccine era. (*Anderson, R.M., 1992*) The proportion of susceptible adults is of sufficient concern that most authorities recommend maintenance of diphtheria immunity by periodic reinforcement with use of Td. (*CDC, 1991*)

### 2.3.12. Safety of Diphtheria Toxoid

Initial efforts to administer booster doses of diphtheria toxoid to older children and adults more than 40 years ago were associated with unacceptably high rates of local and systemic reactions. Such individuals often reacted strongly on Schick testing to both the test and the control material. These reactions appeared to be of the delayed hypersensitivity (tuberculin) type. The solution to the problem of hyper reactivity to diphtheria toxoid lay in three measures. These were enhanced purification of the toxoid to remove extraneous proteins, (*Smith, J.W.G., 1969*) adsorption of the toxoid onto an aluminum salt, and reduction of the amount of diphtheria toxoid per inoculation to 1 to 2 Lf, an amount shown to be sufficient as a booster dose in a number of studies.

The use of Td, rather than monovalent tetanus toxoid, should be standard operating procedure in emergency departments, physicians offices, and other situations in which tetanus-prone wounds are treated. (*CDC, 1991*) Extensive data on adverse reactions after administration of currently available preparations of Diphtheria Toxoid, Adsorbed, are not available because the toxoid is usually administered in combination with tetanus toxoid and, in children, with pertussis vaccine as well. When it is given in combination with pertussis vaccine, local reactions often are ascribed to the pertussis-containing component. In general, the frequency of reported common systemic symptoms (i.e., temperature of 38°C or higher, crying for 1 hour or longer, irritability, drowsiness, loss of appetite, vomiting) and local reactions (i.e., redness, swelling, tenderness) after vaccination with DT or DTaP was comparable. (*Schmitt-Grohe, S., 1997*)

Available data suggest that both diphtheria and tetanus toxoids contribute to the reactogenicity of Td and DT. In some populations, large numbers of previously primed persons develop local reactions and fever in response to diphtheria toxoid, even at low doses. In a small study of Israeli military recruits who had been previously vaccinated in childhood, mild to moderate pain at the injection site was reported by 38% and severe pain by 20% after receipt of a booster dose of 2Lf diphtheria toxoid without tetanus toxoid; limitation of abduction was reported by 8%. Systemic symptoms of mild to moderate or severe weakness were reported by 24% and 9%, respectively, and fever of 38$^0$C or higher was reported by a single subject (<1%).

**2.3.13. Precautions and Contraindications**

There are few contraindications to use of diphtheria toxoid. Neurologic reactions or severe hypersensitivity reactions following a previous dose are considered contraindications to further doses. **(CDCP,1991)** Local side effects alone do not preclude continued use. Vaccination of persons with severe, febrile illness generally should be deferred until recovery, but mild illnesses with or without fever should not preclude vaccination. A fine needle (≤ 23 gauge) should be used for the injection and firm pressure applied to the site, without rubbing, for 2 or more minutes. The patient or family should be instructed concerning the risk for hematoma from the injection.***(CDCP, 2002)***

**2.3.14. Future Prospects**

**2.3.14.1 CRM 197 as a new vaccine against diphtheria.-**

The gene coding for diphtheria toxin has been isolated from phages and studied in details. It has been found to be containing 1850 base pairs in a sequence which has remained constant over last fifty years. Mutagenesis with nitrosoguanidine has resulted into generation of several mutants of this gene, which produce nontoxic proteins that are immunologically related to diphtheria toxin. These have been called as cross-reacting materials (CRMs). These mutants of diphtheria toxin have been designated with different numbers such as CRM 176, CRM 197, CRM 1001, CRM 103 and CRM 107. Their amino acid sequences and changes in activity have been studied in great details in quest of a mutant, which is almost indistinguishable, immunochemically, from diphtheria

toxin. As an immunizing agent it should produce just as high titres of neutralizing antitoxin in rabbits, guinea pigs and finally man as does the toxin itself. CRM 197 contains a single glycine to glutamic acid change in position 52 that makes the fragment A unable to bind to NAD+. It, therefore, is enzymatically inactive and hence nontoxic. Since it is otherwise identical to diphtheria toxin, CRM 197 is the best candidate for a new vaccine against diphtheria.

**2.3.14.1.2 CRM as carrier for polysaccharide vaccines-**

CRM 197 has also been found to be a good carrier for bacterial oligo- and polysaccharides derived from Haemophilus influenzae type B or from Neisseria meningitidis. Therefore, CRM 197 may also be useful in development of polyvalent vaccines. Conjugates between CRM 197 and capsular polysaccharides have been shown to be immunogenic in infants as well as induce a secondary response in adults. The coupling reaction between the protein and the polysaccharide is easier and more reproducible using a purified protein of known primary sequence and physicochemical properties as for CRM 197 than with proteins that have already been randomly modified by previous treatment with formaldehyde, as for tetanus and diphtheria toxoids.

**2.3.14.2 Recombinant vaccines** The tox gene has been isolated, studied, sequenced and also inserted into *E. coli* with the help of various vectors. It has been possible for the gene to express in *E. coli*. For diphtheria, these molecules have been tested for enzymatic activity of fragment A, functionality of fragment B and for the possibility of using them as specific tumour killing agents.

**2.3.14.3 Synthetic antigens:** The diphtherial fragments A and B are joined by a 16 amino acid loop. This has been synthesized and covalently linked to a synthetic poly-DL-alanine-poly-L-lysine polymer. Its injection into guinea pigs evoked the production of antibodies that blocked the enzymatic activity of fragment A. The titres obtained have been somehow low and efforts are being made to develop a more potent synthetic antigen. Attempts are also being made to ascertain the sequence of fragment B of diphtheria toxin so as to synthesize an immunizing agent against intact toxin. The use of these in humans shall require carriers molecules also which may have the drawback of possible sensitization.

**2.3.15 References:**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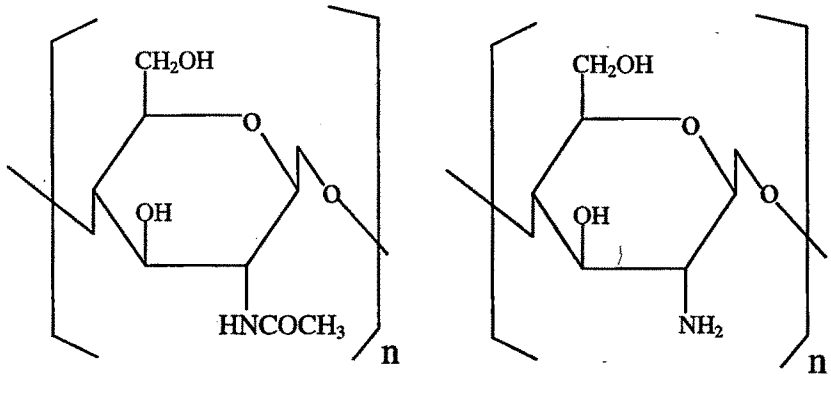
## 2.4 Chitosan

There is growing interest in developing chemical and biochemical processes to obtain and modify biopolymers, and to offer useful technical properties for their application in different fields. One of the latest and most interesting examples is chitin, a cellulose-like bio-polymer found widely in nature as a component of crustaceans, insects, fungi, etc. Chitin is one of the most abundant polysaccharides in nature, second only to cellulose. The molecular skeleton of chitin consists of β-(1,4)-linked glucosamine units with a high degree of N-acetylation, thus masking the basic property of the amino groups and their positive influence on solubility. Principal sourses of chitin are given in **Table 5.** Chitin is, in fact, insoluble in water, dilute acid and alkaline solutions, alcohols, and other common organic solvents. Some solvent systems have been proposed for its dissolution, including N,N-dimethylacetamide, LiCl, N-methylpyrrolidone-LiCl, hexafluoroisopropanol, and a few others. Due to its low solubility, current applications of chitin in its native form are limited in spite of its good tolerability and biodegradability. ***(Ravi Kumar, M.N.V., 2002)***

**Figure 19: Chemical structure of N-acetyl glucosamine (A) and glucosamine (B)**




A B

**Table 5: Principal Sources of Chitin (*Felt, O., 1998*)**

| Organism | | Chitin Content (%) |
|---|---|---|
| Crustacea | Crab | 72.1 |
| | Shrimp | 69.1 |
| | Lobster | 69.8 |
| Insects | True fly | 54.8 |
| | Sulfur butterfly | 64.0 |
| Fungi | Aspergillus niger | 42.0 |
| | Mucor rouxii | 44.5 |

The main use of chitin is for the production of chitosan by a deacetylation reaction, usually obtained in alkaline conditions. Therefore, from a chemical point of view, chitosan is a poly-β-(1,4)-2-amino-2-deoxy-D-glucopyranose, or more simply poly-β-(1,4) glucosamine $(C_6H_{11}O_4N)_n$, with a varying content of N-acetyl groups **(Figure 19)**. Structurally, chitin is a homopolymer with a three dimensional α-helical configuration, stabilized by intramolecular hydrogen bonding. Commercial material contains varying amounts of adsorbed water (7-11 % ) ***(Tomihata, K.,1997)***.

These features, together with the presence of reactive functional groups on the chains, and the biodegradability, biocompatibility and absence of toxicity of the polymer, attracted widespread interest in chitosan as a biomaterial and its use in various fields ***(Paul, W., 2000; Illum, L., 1998)***. Biological properties of chitosan are given in **Table 6.** Its applications include cosmetics, foods, biotechnological preparations and pharmaceutical preparations, mainly as a tablet excipient ***(Singla, A.K., 2001; Felt, O., 1998)***. Chitosan has also been investigated for its hypocholesterolemic action, wound-healing properties and antacid and anti-ulcer activity ***(Felt, O., 1998)***. In addition, its polycationic character gives chitosan the ability to bind strongly to several mammalian cells, leading to many potential uses, including use as a haemostatic and spermicidal agent. These properties have been widely referenced. Characteristics of the polymer, such as the molecular weight (MW) and deacetylation degree (DAD), depend on the processes of native chitin, which cause deacetylation, as well as depolymerization, and greatly influence the properties of pharmaceutical formulations based on chitosan.

**Table 6. Biological Properties of Chitosan (*Hejazi R., 2003*)**

| |
|---|
| Biocompatibility |
| Natural polymer |
| Biodegradable to normal body constituents |
| Safe and non-toxic |
| Haemostatic, bacteriostatic, and fungistatic |
| Spermicidal |
| Anticarcinogen |
| Anticholesteremic |

**2.4 .1 Chemical Properties of Chitosan:-**

Early formulations of chitosan were heterogeneous and poorly characterized, with the DAD ranging from 60-90%. Materials with varying DAD values were defined as

'chitinosans', leaving the term chitosan for a pure poly-glucosamine with 100% DAD; *(Rege, P.R., 1999)* however, this differentiation was not very successful. The term chitosan is suitable for fully or partially N-deacetylated product with a DAD <35% *(Ravi Kumar, M.N.V., 2002)*. Chitosan is now available as well characterized material. Analytical methods for the characterization of chitosan are given in **Table 7.**

**Table 7. Analytical methods for the characterization of chitosan *(Felt, O., 1998)***

| Property | Analytical Method |
|---|---|
| **Molecular Weight** | HPLC |
| | Laser light Scattering |
| | GPC |
| | High-performance GPC |
| **Deacetylation degree** | UV |
| | IR |
| | Tritrimetry |

Deacetylation of chitin can achieve up to 90-95% DAD by heating with concentrated alkaline solution, such as 45% NaOH. The increase of DAD is linearly related to the square root of reaction time suggesting the presence of a diffusion-controlled deacetylation process. Deacetylation lowers crystallinity, increases water absorption, lowers the tensile strength of polymer films, *(Tomihata, K., 1997)* and affects some biologic properties, i.e. the higher the DAD of chitosan, the lower the cell adhesion on films made by this material *(Chatelet, C.,2001)*.

Depolymerization can be obtained by treatment with $NaBO_3$ or $NaNO_2$ solutions containing chitosan dissolved in acetic acid. High MW chitosan (84-90% DAD) can also be directly produced by several species of fungi. A mean MW of $10^6$ is found for chitin, while the deacetylation reaction lowers this value by one order of magnitude *(Ravi Kumar, M.N.V., 2002)*.

Each chitosan subunit contains two (primary and secondary) hydroxyls and one primary amino group at the 2nd position, and represents a suitable substrate for further modifications; the reactivity of depolymerized chitosan is inversely related to MW.

Chitosan is a linear polyamine, whose pKa ranges between 6.1 and 6.3, depending on DAD, concentration, and degree of ionization. The amino groups are readily available for chemical reactions and salt formation with acids. Chitosan is insoluble at alkaline and neutral pH; at acidic pH the amine groups become protonated, and this converts chitosan into a poly-cationic polymer, thus promoting solubility. The solubility depends

on the nature of the acid used to lower pH; inorganic acid promotes solubility to a lesser extent with respect to organic acids. Acids usually employed to dissolve chitosan are formic, acetic, butyric, lactic, glutamic. ***(Leffler, C.C., 2000)*** Chitosan with a low DAD (40%) is soluble up to pH 9, whereas a DAD of about 85% allows solubility only up to pH 6.5. At high DAD and low pH, when the polymer is highly charged, chitosan has an extended and flexible conformation; at lower DAD, due to a lower charge density, the macromolecule adopts a coiled shape. This also occurs in solutions of high ionic strength due to ionic shielding by the electrolyte charges that, at increasing salt concentration, can transform into a salting out effect, leading to the precipitation of the polymer. Chitosan enhances viscosity of the solution, where it is dissolved in acidic media. Viscosity increases with concentration and DAD of the polymer.

Derivatization of the chitosan functional groups modifies the solubility of the polymer and introduces unforeseen properties. (Quarternization of amino groups yields a soluble polymer, whose charge and solubility is independent of pH; ***(Thanou, M.M., 2000)*** N-sulfated chitosan mimics the behavior of glycosaminoglycans, while phosphorylated chitosan reduces adherence of oral bacteria onto tooth surfaces. A water soluble hydroxypropyl derivative was found useful for developing implantable sustained release pharmaceutical forms, ***(Machida, Y.,1986)*** whereas a hydrophobically modified chitosan, containing 4 mol % of n-dodecyl side chains, was prepared as a solubilizing system for insoluble compounds in intermolecular hydrophobic aggregates. A polymer resistant to dissolution under acidic conditions as obtained by reacting chitosan with succinic and phthalic anhydrides.

Chitosan is a complex-forming agent towards heavy metal ions, with a high loading capacity. This behavior can be attributed to the presence of the primary amino groups and the high hydrophilicity and flexibility of the polymer chain. However, development of modified chitosans displayed much higher complexing properties such as -nitriloacetic acid (NTA), -ethylenediaminetetraacetic acid (EDTA), -diethylenetriamine pentaacetic acid (DTPA) conjugates, especially for inactivating metal-dependent enzymes, ***(Bernkop-Schnurch, A.,2000)*** or the preparation of derivatives containing -SH groups, which are able to interact with $Cu^{2+}$ and $Hg^{2+}$. Chemical Properties of chitosan are given in **Table 8.**

Table 8. Chemical Properties of chitosan (*Hejazi R., 2003*)

| |
|---|
| Cationic polyamine |
| High Charge Density at pH< 6.5 |
| Adheres to negatively charged surfaces |
| Forms gels with polyanions |
| High molecular weight linear polyelectrolyte |
| Viscosity, high to low |
| Chelates certain transitional metals |
| Amiable to chemical modification |
| Reactive amino/hydroxyl groups |

**2.4. 2 Properties of Chitossan for Pharmaceutical Use:-**

Its abundance, the possibility to modulate MW and DAD, and hence the physico-chemical properties, make chitosan an attractive material for a variety of purposes: as a flocculating agent in the clarification of waste water and sewage treatment, also for beverages; a chelating agent for detoxification of hazardous wastes; a film generator, to protect fruit crops; an antibacterial and fungicide; for creation of contact lens coating; and the manufacture of wound healing, scaffold materials, etc. The polymeric nature of chitosan, its ability to form gel and films, its cationic character, crystallinity, hydrophilicity, biodegradability, absence of toxicity, and the possibility of sterilization at high temperature, suggested the use of this polymer in pharmaceutical technology for the development of drug delivery systems. In addition, chitosan is considered to be highly degradable and readily excreted in urine; without accumulation in the body after administration. Drugs dispersed in a chitosan matrix may be released by erosion/degradation of the polymer; or, if complexed by the polymer, may also be released from gel by diffusion.

**2.4.2.1 Tablet Excipient:-**

Chitosan can be used as a tablet excipient, for both direct compression ***(Sawayanagi, Y., 1982)*** and standard wet granulation. ***(Ilango, R., 1997)*** Nonetheless, chitosan has not been widely adopted as a pharmaceutical excipient in tablets since virtually all formulations developed to date require the addition of other ingredient to facilitate compression. ***(Rege, P.R., 1999)*** This reflects the fact that commercially available chitosans lack good flow properties and compressibility, ***(Sabnis, S.S., 1997)***

From a technologic point of view, chitosan has been proposed as a diluent and excipient, *(Rege, P.R., 1999; Illum, L., 1998; Dodane, V., 1998),* binder *(Upadrashta, S.M., 1992)* glidant *(Sawayanagi, Y., 1982)* and also used for the production of tablets with controlled release properties. It has been observed that chitosan, at concentrations below 70%, acts as a disintegrant of tablets *(Sawayanagi, Y., 1982)* due to its strong ability to absorb water; at concentrations higher than 5%, chitosan displays a disintegrant power superior to that of starch and microcrystalline cellulose *(Ritthidej, G.C., 1992).* This behavior depends on its concentration; at >50% of the tablet weight an insoluble nonerosion matrix was formed, while at concentrations <33% a fast releasing system was obtained *(Nigalaye, A.G., 1990.)*

The cationic nature of chitosan permits the formation of complexes with negatively charged drugs (or excipients), altering the physico-chemical characteristics of the formulation. A sustained release was evaluated for theophylline in a hydrocolloidal matrix system, where chitosan was coupled to citric acid and polycarboxylic acids. *(Nigalaye, A.G., 1990)* Electrostatic interactions between alginate and chitosan (anionic and cation polyelectrolytes) have been reported as being responsible for controlling the swelling and erosion rate of chitosan tablets in acidic media, and giving the tablets extended release properties.

Grinding chitosan with poorly soluble drugs enhances their dissolution properties *([a]Sawayanagi, Y., 1982; Portero, A., 1998)* by the presence of the hydrophilic polymer. In the case of acidic drugs, a salt forming reaction between the acidic drug and the basic polymer can enhance solubility and dissolution rate, but also promote the formation of a controlling release gel.

With regard to controlling drug release, the most interesting properties of chitosan are its basicity and the interaction with acidic sites of mucosae (mucoadhesivity), or with acidic drugs and excipients, permeation and absorption enhancement, hydrophilicity and consequent water uptake, and the formation of hydrogels.

**2.4.2.1.2 Mucoadhesivity:-**

Improved therapeutic advantages can be obtained by prolonging the residence period of the drug at its critical absorption site, hence adhesion of the delivery system to the absorption membrane represents a prerequisite for an enhanced drug uptake *(Soane, R.J., 1999).* The bioadhesivity of various materials is expressed by their ability to bind

mucopolysaccharides *(He, P., 1988)*. Chitosan has been claimed to act as a bioadhesive, and also as a permeability enhancer. Its mucoadhesive properties can be explained by a multivalent association between the polymer and sialic acid moieties of the mucus gel layer covering the mucosa, as occurs in the case of a univalent association with low MW chitosan *(Hassan, E.E., 1990)*. The attraction between the adhesive and membrane can be through van der Waals forces, hydrogen bonding, or hydrophobic interactions. An optimum adhesion occurs when the adhesive is a polymer whose chains interpenetrate the glycoprotein network of mucin.

Tablets produced from chitosan alone were less mucoadhesive than those prepared with sodium hyaluronate alone, or with both polymers as a complex. *(Takayama, K., 1990)* Chitosan has been employed to coat liposomes to improve the mucoadhesive liposomal dosage form for insulin delivery. *(Takeuchi, H., 1996)*

**2.4.2.1.3 Absorption Promotion:-**

Chitosan is able to promote transmucosal absorption of small polar drugs, *(Illum, L., 1994; Thanou, M., 2001; aThanou, M., 2001)* improving the transport of drugs across mucosal membranes by a combination of bioadhesion and a transient structural reorganization of the tight junctions in the cell membranes, which improves the paracellular route of absorption and allows polar drugs to pass through *(Aungst, B.J., 1996; Schipper, N.G.M., 1997)*. In intestinal epithelial caco-2 cells, chitosan was found to cause a reversible time and dose dependent decrease of transepithelial electrical resistance, *(Dodane, V., 1999)*. In the presence of an excessive mucus layer, the enhanced transport across the membrane is comparatively lower since the polymer cannot reach the epithelium because of size-limited diffusion and/or competitive charge interactions with mucins. *(Schipper, N.G.M., 1999)* The absorption promotion effect does not damage the cell membrane or alter the viability of intestinal epithelial cells.*(Aungst, B.J.,1996)* The preparation of chitosan derivatives has been evaluated in an attempt to overcome its limited solubility and to improve its behavior as an effective absorption enhancer at neutral pH. *(Thanou, M.M., 2000; Thanou, M.M., 2001)*.

Together with these properties, chitosan can offer an additional contribution to enhance permeation. It is a natural metal chelant with a better efficiency than common commercial chelating resins. This activity can be potentially useful in inactivating intestinal metallo-peptidases responsible for presystemic metabolism of orally administered therapeutic peptides. This behavior of chitosan was improved by

conjugation with moieties displaying much higher complexing ability. Chemically modified chitosan can act as an enzyme inhibitor *(Bernkop-Schnurch, A., 2000).*

**2.4.2.1.4 Interaction with Acidic Drug/Excipients:-**

At acidic pHs, chitosan amino groups are protonated, and interact with oppositely-charged drug ions. They can act as release retarding agents, which are useful in the manufacture of prolonged release tablets. This was largely observed in the case of anti-inflammatory acidic drugs, such as salicylic and acetylsalicylic acids, *(Rege, P.R.,1999)* ibuprofen, *(Ilango, R.,1999)* diclofenac sodium, *(Acarturk, F.,1989; Miyazaki, S.,1994)* ketoprofen *(Miyazaki, S.,1994)* and indomethacin *(Aiedeh, K.,1998).* To enhance the interaction, chitosan and the drug can he dissolved in a common solvent, or ground together if the drug is poorly soluble, prior to final formulation.

An increasing chitosan content in a tablet results in a decrease of the release rate of ketoprofen *(Miyazaki, S.,1994).* The extent of the release of salicylic acid to a function of the polymer MW was also explained by the increased number of available amino groups *(Rege, P.R., 1999).* Since cationization of drugs and carriers enhances their cellular uptake by endocytosis, employing cationic polymers or entrapping drugs into cationic carriers, appear to be tools for nonspecific drug targeting to a variety of body districts *(Blau, S.,2000).*

Drug/polymer interactions in the solid state were observed by differential scanning calorimetry for tablets containing chitosan and salicylic acid *(Rege, P.R., 1999).* In the case of the chitosan/ Eudragit® mixture, infrared spectroscopy disclosed an ionic interaction between the amine groups of chitosan and the carboxyl groups of Eudragit®, which provided the system with a new chemical parameter for controlling drug release in the presence of this excipient mixture *(Lorenzo-Lamosa, M.L., 1998).* $^{13}$C-Nuclear Magnetic Resonance spectroscopy provided evidence for deprotonation of the acid by the basic polymer during compression *(Putripipatkhachorn, S., 2001).* This mechanism was also implicated in the role of chitosan as a nutritional supplement. In fact, chitosan affects the lipid transport mechanism in the gut where free fatty acid anions (from hydrolyzed fats), bile salts, cholesterol, and lecithins participate to build up mixed micelles. The positively charged chitosan can bind the acidic components, and hence disturb the whole mechanism of micelle formation that represents a preliminary step to solubilization and absorption of dietary fatty materials. This led to recognition of chitosan as a potential hypocholesterolemic agent since its first applications.

### 2.4.2.2 Hydrogels:-

Hydrogels are hydrophilic polymer networks that can retain a large amount of water. Absorption of water by dried gelified structures usually occurs with a notable increase in volume, as a function of the extent of cross-linkages present in the polymer, and its nature. Gelation occurs in polymer solutions when some parameters are changed, such as concentration, temperature, ionic strength, addition of a nonsolvent or counterions.

Gels can be obtained by dispersing solid $MoO_3$ in a buffered chitosan solution. Cross-linking of polymer occurs by the formation of heavily negatively charged molybdate polyoxyanions; these gels are transparent. thermo-irreversible and can also be prepared at low polymer concentrations. Depending on the ionic strength, these gels are able to swell several times their original size in aqueous solutions. ***(Draget, K.I., 1992)*** Polyelectrolyte complexes are readily formed between chitosan and anionic polymers such as carboxymethyl dextran, poly(carboxylic acid), and alginate. Consistency of the gel formed is affected by the composition of the complex, pH (at least for compounds carrying carboxylate groups) and hence the release of the drug dispersed in the gel. Protonation of these groups at decreasing pH diminished the number of point of electrostatic interactions.***(Ravi Kumar, M.N.V.,2002)*** Chitosan complexes with chondroitin-sulfates and hyaluronic acid have a protective effect against hydrolysis by their specific enzymes.***(Denuziere, A.,1998)*** A novel approach to provide thermally sensitive neutral solutions based on chitosan/glycerophosphate disodium salt combinations was recently described. These formulations possess a physiologic pH and can be held as a liquid below room temperature, but gelify at body temperature. *In vivo*, the liquid formulations turn into gel in situ. This system can be used for tissue engineering applications. In this case, the primary gelation force was attributed to a hydrophobic interaction of neutral chitosan molecules, which can be enhanced by the structuring action of glycerol on water at increasing temperature ***(Chenite, A., 2000).***

The sustaining effect on drug release observed in many cases in the presence of chitosan was attributed to its gel forming ability at low pH, which causes a time-dependent hydration and diffusion process. Gels composed of chitosan are effective matrices for sustained-release formulations of opioid analgesics, capable of providing long-lasting antinociception. Indomethacin and papaverine hydrochloride dispersed in a dried chitosan gel showed a zero order release with 40% indomethacin released at pH 7.4, and 100% papaverine at pH 1 after 24 hours. The same was observed for lidocaine

(lignocaine) from chitosan hydrocolloids and gels ***(Miyazaki, S., 1981)***. In addition, the gels formed from lactic acid and chitosan with high DAD (93%) are more stable in combination with drugs than those prepared with a less deacetylated polymer (43%), due to a high density of electrostatic interactions.

Granules formed from chitosan and indomethacin released the drug faster at pH 7.5 after exposure to acidic pH than if the granules had not been exposed to this low pH. It was thought that this was due to the chitosan swelling and gel formation at this low pH ***(Hou, W.M., 1985)***. Amoxicillin, incorporated in a hydrogel matrix, obtained by cross-linking polyacrylamide-chitosan mixture *in-vitro* had released 77% of the drug after 75 hours in a sustained mode. ***(Risbud, M.V., 2000)***

To minimize undesirable adverse effects, e.g. cardiotoxicity, doxorubicin was conjugated to dextran and encapsulated in chitosan hydrogel nanoparticles. Encapsulation not only reduced the adverse effects, but also improves its therapeutic efficacy in the treatment of solid tumors ***(Mitra, S., 2001).***

The chitosan/glycerophosphate association produces a thermosensitive solution, which gels at 37°C. The gelation rate is dependent on the temperature and DAD; the gel has a highly porous structure after 24-hour exposure to a continuous flow of phosphate buffered saline ***(Noble, L., 1999).***

Chitosan forms hydrogel by covalent cross-linking with glutaraldehyde ***(Jeong, B., 2002)***. Noncovalent cross-linking to form a gel matrix can also be achieved by hydrophobic interactions. The attachment of hydrophobic palmitoyl groups to glycol chitosan, a water soluble chitosan derivative, produced a vesicle-forming polymer palmitoyl glycol chitosan, which is useful in preparing soft, slowly eroding hydrogels suitable for drug delivery by simply freeze-drying an aqueous dispersion of the polymer. ***(Uchegbu, I.F., 1998)*** The freeze-dried material, as examined by scanning electron microscopy, is porous and may be hydrated to up to 20 times its weight in aqueous media without any appreciable change in volume, transforming from an opaque to a translucent solid. The slow erosion of this material in aqueous environments gives a more easily biodegradable and biocompatible material than covalently cross-linked hydrogel. ***(Mitra, S., 2001)***

Hydrogels of N,O-carboxymethyl chitosan released human coagulation factor IX slowly *in vitro* and, when injected subcutaneously, gave prolonged plasma levels compared with those obtained by bolus injection.

Some recent advances in controlled release formulations using chitosan gels, which are responsive to different environmental parameters, have been widely described *(Kost J, 2001).* Chitosan hydrogels, based on interpenetrating polymer network systems, coupled with various macromers or cross-linked hybrid polymers, have been described in detail *(Ravi Kumar, M.N.V., 2002).*

**2.4.2.3 Polymer Micelles:-**

The use of colloidal carriers made of hydrophilic polymers offers a promising alternative for improving the transport of macromolecules across biologic surfaces, *([a]Janes, K.A., 2001)* and a potential drug solubilizer into hydrophobic domains of so-called polymer micelles. These systems appear to be better drug carriers than liposomes or simple surfactant micelles. Polymer micelles can be prepared by attaching alkyl chains to the residue of a hydrophilic polymer; for this use, the polymer-surfactant must be nontoxic, and needs to be biodegradable and metabolizable in living bodies.

The attachment of hydrophobic chains to chitosan yields an amphiphilic polymer capable of self-assembly into vesicles. These polymeric vesicles are found to be biocompatible and hemocompatihle, and capable of entrapping water soluble molecules. Sulfated N-acylchitosan displays solubilization properties towards hydrophobic materials, which increases as the acyl chain length increases. The alkyl chains are directed towards the center whereas the sulfated groups are located on the surface of formation; alternatively alkyl chains can aggregate intermolecularly *(Miwa, A., 1998)* Chitosan was also hydrophobically modified with deoxycholic acid to form self-aggregates in aqueous media able to solubilize DNA plasmid. This approach may find a wide range of applications in gene delivery, as well as general drug delivery applications.

**2.4.2.4 Chitosan Microparticulate Systems:-**

The use of chitosan in microparticulate systems aims to provide kinetic or spatial control of the drug release and improve the bioavailability of many peptide drugs, including insulin, calcitonin, and buserelin. *(Bernkop-Schnurch, A., 2000)* Chitosan properties, including mucoadbesion, biodegradability, *(Muzzarelli, R., 1998)* and permeation-enhancing capabilities for hydrophilic compounds through epithelia, *(Mooren, F.C., 1998)* make it particularly suitable for these purposes. Chitosan microparticulate drug delivery systems have been prepared by different techniques like precipitation, complex

coacervation, chemical cross-linking, and solvent evaporation. The choice of technique is largely dependent on the physico-chemical characteristics of the drug and the desired functional properties of the resulting system.

**2.4.2.4.1 Precipitation:-**

Chitosan precipitation from aqueous acidic solutions is induced in the presence of NaOH *(Nascimento, A., 2001)* or sodium sulfate *(Stolberg, J., 1999; Hejazi, R., 2002; Sezer, A.D., 1999)*. A spherical form is obtained by adding the chitosan solution drop wise to the alkaline solutions or adding the alkaline solution to the chitosan solution containing polyoxyethylene sorbitan monooleate (Tween™80)1% w/v *(Nascimento, A., 2001; Stolberg, J., 1999)*. The product obtained by this method is a polyion complex, poorly soluble in aqueous systems. Chemical cross-linking has been carried out after precipitation by placing the gelled microspheres in contact with a glutaraldehyde 2.5% w/v solution. *(Nascimento, A., 2001)* In other cases, only freeze-drying *(Stolberg, J., 1999; Hejazi, R., 2002)* is carried out to obtain the final dry product. The drug may be incorporated in the aqueous solution of chitosan before precipitation, or incubated with the microspheres formed in aqueous *(Mooren, F.C., 1998; Stolberg, J., 1999)* or hydro-alcoholic solutions *(Nascimento, A., 2001)*. The microspheres obtained by simple chitosan precipitation without further cross-linkage dissolved at acidic pH, thus proving suitable systems for the site-specific delivery of tetracycline in the stomach which is particularly useful in the presence of *Helicobacter pylori*. Moreover, microspheres obtained by simple precipitation have been evaluated as carriers of hydrophilic drugs, such as prednisolone sodium phosphate, across the intestinal epithelial barrier. It was shown that the uptake of the drug was improved across the intestinal epithelium, probably by increasing the transport of the hydrophilic drug through the tight junctions of the epithelium *(Mooren, F.C., 1998)*. Microspheres of chitosan obtained by precipitation and cross-linked with glutaraldehyde were subsequently grafted with polyacrylic acid and loaded with aspirin (acetylsalicylic acid), by soaking in a hydro-alcoholic mixture. These microspheres showed high efficiency of impregnation and a sustained release of the drug, both at acidic and alkaline pH *(Nascimento, A., 2001)*.

**2.4.2.4.2 Complex Coacervation:-**

The formation of a precipitate following the complexation between two oppositely charged polymers produced an ionic cross-linkage. Chitosan solubilized in acidic aqueous solution is generally added to a solution containing polyanions, such as

alginates,*(Sezer, A.D., 1999; Vandenberg, G.W., 2001)* sodium hyaluronate,*(Lim, S.T., 2000)* or tripolyphosphate *(Fernandez, U.R., 1999; Shu, X.Z., 2001; Ma, Z., 2002; Dyer, A.M., 2002).*The aqueous solution may be extruded drop wise through a needle into the polyanion solution. The complex coacervation may also be carried out by the emulsion technique, where chitosan is solubilized in the aqueous phase of water in oil (w/o) emulsion, *(Shu, X.Z., 2000)* and the polyanion solution added drop wise while stirring. The particles formed by complex coacervation do not dissolve in an aqueous environment; so further stabilization by chemical cross-linkage is not required. This method is particularly suitable for incorporating molecules sensitive to organic solvents, temperature, pH, etc. Complex coacervation with chitosan has been used to evaluate the incorporation efficiency and release of insulin, *(Fernandez, U.R., 1999; Ma, Z., 2002; Dyer, A.M., 2002)* cyclosporine, *(Janes, K.A., 2001)* and macromolecular model substances such as dextran *([a]Sezer, A.D., 1999)* or bovine serum albumin. *(Vandenberg, G.W., 2001)* Chitosan microparticles obtained in the presence of tripolyphosphate provided insulin loading of about 55%. *(Fernandez, U.R., 1999)* In contrast, chitosan nanoparticles, prepared by complex coacervation of chitosan glutamate in the presence of pentasodium tripolyphosphate, did not improve the absorption-enhancing effect of chitosan in solution or powder form, while chitosan powder was the most effective formulation for nasal delivery of insulin in the sheep model *(Dyer, A.M., 2002).* The insulin association efficiency was measured in chitosan nanoparticles prepared by complex coacervation with tripolyphosphate. This parameter is sensitive to the formulation pH. The highest association efficiency was measured at pH 6.1, close to the pH value for the isoelectric point (pI) of native insulin and the pKa of chitosan. This association was attributed to physical absorption of insulin, through hydrophobic interactions with chitosan, allowing a rapid insulin release in aqueous media. The association efficiency obtained at pH 5.3 was less than half that obtained at pH6.1, but the association at pH 5.3 appeared to be based on stronger interactions, because the release of insulin was pH-dependent and the recovery was <25%, even upon disintegration of the chitosan matrix *(Ma, Z., 2002).*

A hydrophobic peptide, cyclosporine, was loaded into chitosan nanoparticles by solubilization in an acetonitrile/water mixture (1:1), and incorporation of this solution into chitosan solution or tripolyphosphate solution before coacervation. The nanoparticles obtained had high loading and the *in vitro* release studies revealed a fast release during the first hour, followed by a more gradual drug release, during a 24 hour

period. *In vivo* experiments, aimed at evaluating these nanoparticles as extraoccular drug delivery systems, showed that following topical instillation of the nanoparticles, therapeutic concentrations were reached in external ocular tissues lasting at least 48 hours, while maintaining negligible or undetectable cyclosporine levels in inner ocular structures, blood, and plasma. These levels were higher than those obtained following instillation of a chitosan solution containing cyclosporine or an aqueous cyclosporine suspension.

Chitosan nanoparticles formed by complex coacervation were evaluated as carriers of doxorubicin ***(Janes, K.A., 2001).*** Doxorubicin was complexed with the polyanion dextran sulfate to mask its positive charge and improve its entrapping into the chitosan nanoparticles. The nanoparticles obtained were characterized by loading levels of up to 4.0% by weight. The evaluation of the activity in cell culture revealed that doxdrubicin was not released in the medium, but entered the cells associated with the nanoparticles. The cytostatic activity of the nanoparticles was similar to that of the free drug, indicating that the drug from the nanoparticles is delivered in the cells.

**2.4.2.4.3 Chemical cross-linking:-**

Chemical cross-linking consists of the formation of covalent bonds between the free amino grouped of chitosan and glutaraldehyde or formaldehyde; genipin ***(Mi, F.L., 2002)*** and ascorbyl palmitate ***(Aiedeh, K., 1997)*** have also been used as chemical cross-linking agents. The microspheres may be obtained by spray drying aqueous solutions or dispersions of chitosan containing cross-linking agent ***(Lim, S.T., 2000; [a]He, P., 1999; Ganza Gonzalez, A., 1999)*** or spray drying aqueous solution of chitosan and subsequently dispersing the microspheres obtained into glutaraldehyde or genipin hydroalcoholic solutions ***(Mi, F.L., 2002).*** The microspheres may also be prepared in emulsion, where chitosan is solubilized in the acidic aqueous phase of w/o emulsion and the cross-linking agent is added to the emulsion. Following the partition of the cross-linking agent towards the aqueous phase, chitosan cross-linkage takes place. Depending on the partition characteristics of the cross-linker, cross-linkage may take place in the whole aqueous phase, providing microspheres, or at the interface of the w/o emulsion, providing microcapsules (as is the case of ascorbyl palimatate) which due to its amphiphilic properties, gravitate towards the emulsion interface. The cross-linkage in the whole aqueous phase may be inappropriate in the presence of peptide drugs as the cross-linking process may damage it. In this case, the drug may be loaded into the pre-

formed microspheres by soaking in the presence of an aqueous phase. However, this procedure limits the encapsulation efficiency, due to the high molecular dimension of the peptide drugs. Insulin loading in chitosan microparticles has been efficiently carried out by an interfacial cross-linkage technique in the presence of ascorbyl palmitate *(Aiedeh, K., 1997)*. The microparticles obtained were characterized by high loading levels of insulin, a complete release of the drug in about 8 hours, a release rate almost constant over time.

**2.4.2.4.4 Solvent Evaporation:-**

Using solvent, the evaporation of water from chitosan acidic aqueous solutions provide chitosan precipitation in the form of the salt present in the solution. Therefore, the systems obtained are readily soluble in aqueous solutions especially in the acidic medium. The drug to be loaded in the microspheres is generally solubilized or dispersed in the aqueous phase containing the chitosan. In the w/o emulsification solvent evaporation technique, or dry-in-oil emulsion method, microspheres are obtained by water evaporation from w/o emulsions containing sorbitan monooleate (span 80) *(Lim, S.T., 2000)* or sorbitan monolaurate (Span 20) *(Perugini, P., 2000)* as the emulsifying agent, and chitosan in the inner aqueous acidic phase. Evaporation may be obtained by maintaining the temperature at 40°C for 12 hours, *(Lim, S.T.,2000)* or at 55°C under reduced pressure for 10 hours, *(Perugini, P., 2000)* when the solvent is evaporated completely, the microspheres are washed with hexane *(Lim, S.T.,2000)* or petroleum ether to remove excess oil, filtered, and dried *(Perugini, P.,2000)*. Microspheres prepared by simple evaporation did not provide sustained release of gentamicin sulfate as the release took 50% longer from hyaluronic acid microspheres, *(Lim, S.T., 2000)* and the glycolic acid release was not controlled, even after cross-linking obtained by adding glutaraldehyde aqueous solution into w/o emulsion before solvent evaporation *(Perugini, P., 2000)*. The solvent evaporation may also be obtained by spray drying aqueous acidic solutions of chitosan. In this case, the use of the oil phase and the emulsifying agent is not required, thus avoiding the washing procedure after solvent evaporation *(Giunchedi, P., 2002)*. Chemical cross linkers, such as formaldehyde or glutaraldehyde, may be added to the chitosan aqueous solution before spray-drying *([a]He, P., 1999; Ganza Gonzalez, A., 1999; He, P., 1999)* to obtain microspheres insoluble in the aqueous phase, and thus suitable to sustain the drug release. Microspheres prepared with more than 15% formaldehyde (by weight with respect to the polymer)

showed good release control of metoclopramide *(Ganza Gonzalez, A., 1999)*. The release of cimetidine, famotidine, and nizatidine from microspheres cross-linked with either glutaraldehyde or formaldehyde, was fast and accompanied by a burst effect; the increase in the cross-linking extent reduced the release rate *([a]He, P.,1999)*. The spray-drying procedure has also been used with oil in water or water in oil in water emulsions containing chitosan in the aqueous phase to slow down the release of cimetidine and famotidine, which was almost instantaneous from the chitosan microspheres obtained by spray-drying the aqueous solutions of the polymer *([a]Sezer, A.D., 1999)*.

Glutaraldehyde is toxic and it is important to evaporate its residual content in the final microspheres, when it is used as a cross-linker for in-vivo formulations.

**2.4.3. Chitosan and Routes of Drug Administration:-**

Chitosan was proposed as a drug carrier for mucosal administration in ocular, buccal, nasal, gastrointestinal, and vaginal-uterine therapy, based on its biadhesivity and biodegradability.

**2.4.3.1 Ophthalmic Delivery:-**

Due to their elastic properties, chitosan hydrogels offer better acceptability, with respect to solid or semisolid formulations, for ophthalmic delivery, and in many cases they can be administered as a liquid and then transformed into gel after the dose has been applied.

Ophthalmic chitosan gels improve adhesion to the mucin, which coats the conjunctiva and the corneal surface of the eye, and increases precorneal drug residence times, slowing down drug elimination by the lachrymal flow. In addition, its penetration enhancement has a more targeted effect and allows lower doses of the drug *(Kaur, I.P., 2002)*.

In formulations containing tobramycin as a therapeutic agent, chitosan was well tolerated on the corneal surface. Gamma scintigraphic data showed that the clearance of the formulation, labeled with $^{99m}$Tc-DTPA, was significantly delayed in the presence of chitosan with respect to a commercial reference collyrium, obtaining a 3-fold increase in corneal residence time *(Felt, O., 1999)*.

The duration of efficacy of the antibacterial ofloxacin was increased from about 25 minutes to 46 minutes by a high MW (1930kd) and low DAD (50%) chitosan, at a concentration of 0.5% wt/v, when compared with a control. A similar performance was

achieved by a low MW (580kd) chitosan, but at a concentration of 1.5% wt/v in the case of tobramycin *(Felt, O., 2001).* The *in vitro* dissolution profile of acyclovir from chitosan microspheres was slower than that for the raw drug, and *in vivo* ocular administration of the same system resultes in prolonged high concentrations of the drug and increased the area under the concentration-time curve values *(Genta, I., 1997).*

**2.4.3.2 Nasal Delivery:-**

Chitosan, in the form of powder, or 0.5-1.0% w/v solution, improved absorption of many drugs, including peptides and proteins, or the immunologic response of vaccines, when administered intranasally *(Draget, K.I., 1992).* In fact, the nasal membrane is highly vascularized and has a large surface area, readily accessible for drug absorption, due to the presence of turbinates. Morphine administered intranasally to humans, as a simple solution, is only absorbed to a limited degree, with a bioavailability in the order of 10%, compared with intravenous administration. Morphine associated with chitosan was rapidly absorbed, with a $t_{max}$ of >15 minutes and a bioavailability of nearly 60%, and can result in a noninjectable opioid formulation capable of offering patients rapid and efficient pain relief. Morphologic and histologic evaluation of the rat nasal mucosa following a 2-week daily administration, indicated that chitosan produced only mild to moderate irritation *(Dyer, A.M., 2002).* The nasal instillation of insulin was largely examined to improve its systemic absorption *(Hinchcliffe, M., 1999).* In the sheep model, an insulin/chitosan association for nasal delivery caused a reduction of plasma glucose level to 43%, compared with 83% value in the absence of chitosan. Similar bioavailability increases were obtained with other peptides. The effect of chitosan on insulin absorption across the nasal mucosa is concentration dependent, with the optimal efficacy obtained for concentrations higher than 0.2 and 0.5% in rats and sheep, respectively *(Illum, L., 1994).* Studies using the powdered form of chitosan were less successful due to its slow dissolution *(Schipper, N.G.M., 1997).*

Chitosan nanoparticles, with a size in the range of 300-400 nm, enhanced the nasal absorption of insulin to a greater extent than when in solution with chitosan. The amount and MW of chitosan did not have a significant effect on insulin response *(Illum, L., 1994; Fernandez, U.R., 1999).*

Poly (lactic acid-glycolic acid) nanoparticles, mucoadhesive and stabilized to lysozyme due to the presence of chitosan, also enhanced the nasal transport of encapsulated tetanus toxoid, providing high and long-lasting immune responses *(Vila, A., 2002).*

Biodegradable microparticles containing gentamicin were prepared using chitosan hydroglutamate, hyaluronic acid, and a combination of both polymers. These formulations, when administered nasally via an insufflator, enhanced the bioavailability of gentamicin, compared with a simple nasal solution in vivo. When hyaluronic acid and chitosan hydroglutamate were combined in a hyaluronic acid chitosan hydroglutamate formulation, the polymers appeared to improve the absorption of incorporated gentamicin synergistically, compared with the individual polymers ***(Lim, S.T., 2002).***

**2.4.3.3 Buccal Delivery:-**

Buccal drug delivery represents a versatile application, not only in the treatment of local infections, such as stomatitis, periodontal diseases, or mucosal lesions, but also as a topical route for systemic delivery of therapeutic peptides, avoiding hepatic first-pass metabolism, acidity, and protease activity encountered in the gastrointestinal tract. In these cases, adhesion developed by a chitosan hydrogel appears to be suitable for prolonging the residence period of the pharmaceutical form, and improving the therapeutic effect. ***(Chatelet, C., 2001)*** Also in this case, gamma scintigraphy assessed the limited clearance of a $^{99m}$Tc radiolabeled formulation containing 10% chitosan from the periodontal pocket.

Gels of chitosan (at 1 or 2% concentration) containing 0.1 or 0.2% chlorhexidine gluconate, release the antifungal agent chlorhexidine gluconate for 3 hours; a prolonged release was also observed with film formulations. No lag-time was observed in the release of chlorhexidine gluconate from either gels or films. The highest antifungal activity was obtained with 2% chitosan gel containing O.1 % of the drug. ***(aSenel, S., 2000)*** The loading of chlorhexidine into chitosan to improve the anti-microbial activity of the drug was also confirmed by different formulations. ***(Giunchedi, P., 2002)*** An additional antifungal effect was achieved since chitosan also inhibits the adhesion of Candida albicans to human buccal cells.

A 0.5% w/v solution depressed the fungal adherence on a surface according to the sequence: low MW chitosan >phosphorylated chitosan > amorphous chitosan >carboxymethyl chitosan.

The inhibitory effect on the adherence of oral fungi onto tooth surfaces was similar. ***(Sinha, V.R., 2002)*** The effect of chitosan as a permeabilizer was determined by measuring the flux of Transforming Growth Factor (TGF-β□ across porcine oral mucosa

in an *in vitro* system ***(Senel, S., 2000).*** The buccal permeation enhancement was also observed for hydrocortisone, a commonly used topical oral anti-inflammatory agent. ***(Senel, S., 2001)***

**2.4.3.4 Oral Delivery:-**

Oral ingestion of drugs is the most common route of administration, but unfortunately the gastrointestinal tract represents a barrier for absorption of peptides and proteins. Chitosan, displaying enzyme inhibiting and absorption enhancing activity, appears to be a suitable adjuvant for this purpose, and chitosan derivatives have been evaluated to bypass the gastric medium and operate at neutral pH values, such as those found in the intestinal tract ***(aBernkop-Schnurch, A.,2001).*** Trimethyl chitosan chloride, at different degrees of quaternization, increases the permeation and/or absorption of neutral and cationic peptide analogs across intestinal epithelia. Monocarboxymethylated chitosan, a polyampholytic polymer able to form visco-elastic gels in aqueous environments or with anionic macromolecules at neutral pH values, appears to be less potent than the quaternized derivative. Nevertheless, mono-carboxymethylated chitosan was found to increase the permeation and absorption of low MW heparin across intestinal epithelia. The mechanism by which trimethyl chitosan chloride enhances intestinal permeability is similar to that of the protonated chitosan; it reversibly interacts with components of the tight junctions, widening the paracellular routes, without permanent damage to the cell membrane and without altering the viability of intestinal epithelialial cells ***(Thanou, M., 2001).*** A sustained or delayed ampicillin release can be provided by delivery systems containing such a polymer ***(Chandy, T., 1993).***

Bioadhesive tablets for intraoral drug delivery were prepared, by directly compressing a drug with a mixture of chitosan and sodium alginate in weight ratios of 4:1, 1:1 and 1:4, and the adhesion and release characteristics of the prepared systems were evaluated *in vitro* and *in vivo*. The plasma concentration curves for the tablet with a 1:4 chitosan/alginate ratio showed a sustained release 3 hours after administration ***(Miyazaki, S., 1994).*** The percentage of ampicillin release, embedded in a chitosan matrix was much higher in an acidic solution compared with the phosphate solution, probably due to the gelation properties of the matrix at low pH ***(Chandy, T., 1993).*** The release rate of the drug from the chitosan matrix was slower for beads than granules, thereby offering the possibility of modifying the formulation to obtain the most convenient oral sustained delivery system.

**2.4.3.5 Colon Delivery:-**

The rationale for the development of a polysaccharide-based delivery system for the colon is the presence of a large number and variety of polysaccharidases, such as β-D-glucosidase, β-D-galactosidase, amylase, pectinase, xylanase, β-D-xylosidase, dextranase, etc. Therefore, pharmaceutical forms that are designed to cross the gastrointestinal tract unaltered, can release the active agent at the colon level coupled to a polysaccharide, such as chitosan, and are thus proposed as potential colon specific drug carrier systems *(Sinha, V.R., 2001).* Chitosan can be modified by reacting with succinic and phthalic anhydride to prepare matrices resistant to dissolution under acidic conditions. Improved drug release profiles under basic conditions suggested that these matrices are suitable for colon-specific delivery of orally administered drugs.

A dispersed system, composing of an active ingredient reservoir and the outer drug release-regulating layer in hydrophobic polymer, proved useful for colon-specific drug delivery; the outer layer contained chitosan powder. In this case, an additional outer enteric coating was necessary to prevent drug release in the stomach, since chitosan dispersed in the layer dissolves easily under acidic conditions. Resulting enteric-coated capsules reached the large intestine within 1-3 hours after oral administration, and degraded at the colon level in beagle dogs *(Blau, S., 2000).*

Systems for colon delivery containing acetaminophen, *(Sinha, V.R., 2002)* mesalazine (5-ASA), *(Tozaki, H., 2002)* sodium diclofenac,*(Orienti, I., 2002; Gonzalez-Rodriguez, M.L., 2002)* and insulin have been studied. Chitosan capsules containing insulin entered the colon 8 hours after administration, as evaluated by transit time experiments, starting the hypoglycemic effect from that time *(Tozaki, H., 1997; [a]Zhang, H., 2002)*

Potential drug delivery at the level of the colon can be evaluated in vitro from release in a slightly alkaline medium, such as that present in the colon environment, or from degradation of the pharmaceutical form in the presence of rat cecal and colonic enzymes *(Zhang, H., 2002).* Degradation depends on both the MW and DAD of the chitosan sample; samples with a lower MW and DAD are more susceptible substrates *(Macleod, G.S., 1999; Shimono, N., 2002).*

**2.4.3.6 Vaginal Delivery:-**

Chitosan, modified by the introduction of thioglycolic acid to the primary amino groups of the polymer, embeds clotrimazole, an imidazole derivative widely used for the

treatment of mycotic infections of the genitourinary tract. By introducing thiol groups, the mucoadhesive properties of the polymer were strongly improved and this resulted in an increased residence time of the vaginal mucosa tissue (26 times longer than the corresponding unmodified polymer), guaranteeing a controlled drug release in the treatment of mycotic infections *(Kast, C.E., 2002).*

**2.4.3.7 Transdermal Delivery:-**

Chitosan finds applications in transdermal drug delivery for preparing transdermal devices, implantable dosage forms, and topical formulations.

The applications of chitosan in preparing transdermal drug devices are related to its film-forming property. The kinetic characteristics, e.g. permeability, are affected by the cross-linking and thickness of the membranes. Chitosan gel was used as a drug reservoir in permeation-controlled transdermal drug delivery systems for propranolol hydrochloride *(Thacharodi, D., 1995).* Chitosan membranes with different permeability to the drug, obtained by controlled cross-linking with glutaraldehyde, were used to regulate the drug release in these devices. A pore mechanism transport for water-soluble drugs was suggested, while a partition mechanism is expected for hydrophobic solutes. The biologic properties of chitosan (biodegradable, nontoxic, sterilizable, harmless degradation product polymer) make it suitable in developing implantable dosage forms (microspheres, tablets, etc.), which can be enzymatically degraded. The prolonged residence time of the form allows a longer local or systemic supply of the active agent. Its localization, e.g. inside a solid tumor mass, offers the advantage of a local efficient release, thereby reducing adverse effects. The partition of the drug between the skin and a topically applied hydrogel matrix is an important parameter in the permeation process; chitosan was also tested from this point of view.

In vitro capsaicin permeation showed higher levels in cationic chitosan and anionic carboxymethyl cellulose hydrogels than in cream bases *(Wang, Y.Y., 2001).* Nonivamide, which has a similar chemical structure and pharmacologic activities as those of capsaicin, inserted into chitosan hydrogels, produced higher levels of *in vitro* nonivamide permeation and skin distribution; the *in vivo* effects depended on dose and duration of the topical application *(Fang, J.Y., 2002).*

Topical systems for controlled delivery of glycolic acid were prepared to optimize the cosmetic properties of the acid, thereby reducing its adverse effects. Two types of

liposomes were investigated: the addition of chitosan into the lipidic bilayer during liposome preparation; and coating of already formed liposomes with chitosan. The results showed that liposomes always modulated glycolic acid release and that the best condition to achieve this control was obtained by the liposomal systems in which the glycolic acid/lipid molar ratio was 5:1. On the contrary, chitosan microspheres are not able to control glycolic acid release, even after cross-linking *(Perugini, P., 2000)*. The role of several polymers was evaluated as an adjuvant to design a transdermal delivery system for the treatment of cutaneous leishmaniasis. Within in vitro skin perfusion studies of berberine incorporated into the chitosan hydrogel to prepare ointments, *(Tsai, C.J., 1999)* only trace amounts of the drug permeated through rat skin due to its low oil-water partition coefficient. Anionic polymers in the test solutions decreased the skin permeation of morphine, whereas chitosan increased it, compared with the polymer-free solution *(Hosoya, O., 1998)*. On the other hand, cationic and anionic polymers in test solutions respectively decreased and increased the skin permeation of salicylic acid from the same enhancing system containing sodium salicylate. These contrasting results can be attributed to the interaction between the drug and polymer, carrying opposite charges; electrostatic interactions change the skin permeation of the drug *(Thacharodi, D., 1995)*.

### 2.4.4. Specific Chitosan Applications:-

#### 2.4.4.1 Radiopharmaceuticals:-

A radiopharmaceutical contains a radioactive source and is administered either for diagnostic or therapeutic purposes. In both cases, it needs precise localization in a preselected body district and organ, and a sufficiently long period of residence. Since modern radioactive sources for external imaging are radioisotopes of transition metals, the complexing ability of chitosan represents a promising tool for preparing macrocomplexes as radiopharmaceuticals; thus far experimentation has been limited to a very few cases.$^{166}$Ho-chitosan complex is a radiopharmaceutical drug for cancer therapy. The radioactivity is retained at the administration site after either intrahepatic or intratumoral administration to rats or tumor-transplanted nude mice; this was attributed to the chemical form of chelate of the radioisotopes with chitosan, which demonstrate a positive role as a carrier. Similarly, following the intra-articular injection of $^{166}$Ho-chitosan complex for a localized treatment of knee synovitis in rheumatoid arthritis, a

gamma scan study indicated that most of the injected radiochemical was localized within the injected joint cavity, and extra-articular leakage was negligible 24 hours after the injection. Also, in this case the $^{166}Ho$ radiopharmaceutical was retained at the site of administration in the form of chelate with chitosan.

The accumulation of gadolinium loaded as gadopentetic acid in chitosan nanoparticles (Gd-nanoCPs), designed for gadolinium neutron-capture therapy for cancer, was 100-200 times higher in cultured cells than a commonly used magnetic resonance imaging contrast agent. These findings indicated that Gd-nanoCPs had a high affinity to the cells, probably contributing to the long retention of Gd in tumor tissue, and leading to a significant suppression of tumor growth.

Radiophannaceuticals containing $^{99m}Tc$ were employed to assess localization and residence of pharmaceutical forms by means of adhesive properties of chitosan present in such formulations *(Felt, O., 1999).*

**2.4.4.2 Gene Delivery:-**

In efficient gene delivery, plasmid DNA is introduced into target cells. For this purpose it is important to develop non viral gene delivery vectors to transfect the target cells in vivo *(Borchard, G., 2001)* The two major types of vectors that have been suggested are cationic phospholipids and cationic polymers, which, because of their permanent cationic charge, are able to interact and form complexes with negatively charged DNA. The possibility of carrying plasmid DNA would exploit the full potential of chitosan, which may be a suitable material for efficient nonviral gene and DNA vaccine delivery. *(Illum, L., 2001; Mao, H.Q., 2001)* Chitosan can form polyelectrolyte complexes with plasmid DNA, where DNA is protected against nuclease degradation, as reported in the case of *Mycobacterium phlei* DNA; it inhibits cancer cell division, but is susceptible to degradation by deoxyribonuclease. Incorporation of DNA within chitosan nanoparticles significantly decreased this degradation. *(Kabbaj, M., 2001)* Microcapsules containing calf thymus DNA and prepared by interfacial polymerization of chitosan, and alginate microspheres formed by emulsification/internal gelation, were recovered intact from rat feces following gastrointestinal transit. This is the first report of microcapsules or microspheres containing DNA being passed through the gastrointestinal tract with the potential for substantial recovery.

Chitosan-DNA complexes can be prepared mixing a solution of both components; the size of the final complex depends on polymer MW. At physiologic pH, a large complex is found when two amino groups of chitosan are available for one DNA phosphate. Aggregation of the complexes occurs when they are electrically neutral, and in these conditions full retardation of plasmidDNA release is observed.

A coacervation process was used to prepare chitosan-DNA nanoparticles. At an amino-to-phosphate group ratio of between 3 and 8, and a chitosan concentration of 100 µgm/ml. the size of particles was in the range of 100-250 nm with a narrow distribution and a composition of 35.6 and 64.4% by weight for DNA and chitosan, respectively. The transfection efficiency of chitosanDNA nanoparticles was found to be cell-type dependent *(Borchard, G., 2001).*

Trimethyl chitosan oligomers, of 40-50% quaternization degree, proved superior to simple chitosan in transporting model cells. Chitosan, equipped with moieties (lactose, galactose) for specific cell interaction, displays an efficiency which is dependent on the cell line used *(MacLaughlin, F.C., 1998).*

**2.4.4.3 Peptide Delivery:-**

The oral administration of peptide drugs represents one of the greatest challenges in pharmaceutical technology. To gain sufficient bioavailability of these therapeutic agents, various barriers, including the mucus-layer barrier, enzymatic barrier, and membrane barrier, have to be overcome. The protective and mucoadhesive properties of peroral peptide delivery systems containing chitosan are more important for rapidly degradable peptides in the intestinal environment, whereas the permeation enhancing effect is less important for peptides of a molecular size smaller than 30Å *(Bernkop-Schnurch, A., 2000).*

When the peptide drug is homogenized with chitosan or derivatives and is directly compressed in a tablet without any protection, none of the positive properties of chitosan discussed in above sections are displayed. Dissolution physiologic medium (containing protease) penetrates the polymeric network of the hydrophilic matrix and the embedded peptide will degrade unless chitosan is used with an improved inhibitory property. According to this strategy, a trimethylate chitosan was deemed useful tool for delivery systems targeting the large intestine and colon. The covalent attachment of thiol moieties on these polymers leads to improved mucoadhesive and permeation-enhancing

properties, and the conjugation of enzyme inhibitors enables the matrices to provide protection for peptide drugs against enzymatic degradation. Thioglycolic acid, covalently linked to chitosan by an amide bond, displays improved permeation-enhancing properties, with respect with chitosan hydrochloride alone or in a mixture with unbound acid ***(Kast, C.E., 2001).***

As an additional adjuvant to permeation enhancement, chitosan can be modified to inhibit intestinal proteases responsible for the presystemic metabolism of poly(peptide) drugs. The immobilization of chelants on the chitosan chain improved the inhibition of various metallo-peptidases. A DTPA conjugate displays ***(Bernkop-Schnurch, A., 2000)*** higher complexing ability than an EDTA analog, and its substructure makes it swellable in a large pH range. However, the inhibitory effect of chitosan towards peptidases was less pronounced, due to the lower number of substitutions obtained with DTPA for steric reasons.

Chitosan particles, powders, and solutions are also potential vehicles for mucosal vaccine delivery. The polymer, in the form of microparticles and nanoparticles, encapsulates large amounts of antigens, such as ovalbumin, diphtheria toxoid, or tetanus toxoid. Such systems are promising candidates for mucosal vaccination. The striking advantage of mucosal vaccination is the production of local antibodies at the sites where pathogens enter the body. Because vaccines alone are not sufficiently taken up after mucosal administration, they need to be co administered with penetration enhancers, adjuvants, or encapsulated in particles. In addition, after co administering chitosan with antigens in nasal vaccination studies, both mucosal and systemic immune responses were strongly enhanced ***(a van der Lubben, I.M.,2001; van der Lubben, I.M., 2001).***

A double inhibition towards serum proteases and metallopeptidases was obtained by conjugating, on the same chitosan chain, EDTA and an enzyme inhibitor. The final material also displays strong mucoadhesive properties. The immobilization of inhibitors such as antipain, chymostatin, elastatinal, and Bowman Birk inhibitor provide a protective effect towards pancreatic serine proteases, whereas covalently attached complexing agents such as EDTA, guarantee the inactivation of membrane bound Zn-dependent peptidases, as well as carboxypeptidase A and B. Since the inhibition of these enzymes strongly improves the bioavailability of orally administered peptide drugs, chemically modified chitosans appear promising auxiliary polymers. ***(Tozaki, H., 1997; Bernkop-Schnurch, A., 2001)***

Chitosan glutamate effectively enhances the transport of macromolecules across a buccal TR146 cell culture, a model of the buccal epithelium, and is perhaps a promising vehicle for peptide and protein buccal administration.*(Portero, A., 2002)* Quaternized chitosan derivatives have been evaluated to overcome the limited solubility and effectiveness of chitosan as a peptide absorption enhancer at neutral pH values such as those found in the intestinal tract *(Thanou, M.M.,2000).*

Both the free amine and acid salt forms of chitosans are effective in enhancing the nasal absorption of [D-Arg(2)]-Kyotorphin, an enzymatically stable opioid dipeptide; the polymer has been proposed as a well tolerated and effective nasal absorption enhancer of peptide drugs.

Insulin release from alginate-chitosan and alginate-chitosan-glutaraldehyde beads was studied in artificial gastric (pH 1.2) and intestinal (pH 7.5) fluids. The beads remained stable and the undegraded insulin seemed to be sufficient for physiologic conditions. This system could be adopted for oral delivery of insulin.

The mucoadhesive properties of DL-lactide/glycolide copolymer nanospheres containing elcatonin were evaluated by measuring the nanospheres adsorbed in a rat everted intestinal sac. The chitosan-coated nanospheres showed higher mucoadhesion than other polymer-coated nanospheres, and significantly reduced the blood calcium level compared with elcatonin solution and uncoated nanospheres. The reduced calcium level was sustained for a period of 48 hours.

Because of the peculiar advantages offered by its chemical and biologic properties, chitosan has been widely investigated as a drug carrier for many possible routes of administration. Chitosan was found to be nontoxic, metabolized by lysozymes, poorly allergenic, and to have a high lethal dose (50% of lethal dose [LD50] of 16g/kg). Hence, it was also examined for a variety of biomedical applications, which merit some mention. Orally administered chitosan binds to fats in the intestine. Its dietary supplementation was suggested for weight control and as a consequence, as an atherosclerosis inhibitor and for the reduction of urea and creatinine serum levels. It is also thought to have positive effects in the treatment of renal failure. The ability of chitosan to reduce the ulcerogenic activity of some nonsteroidal anti-inflammatory drugs was attributed to the solubility and gel forming ability of chitosans in an acidic medium, which exerts a protective effect on the stomach mucosa. Chitosan was reported to have a haemostatic

effect and its use as a dressing material which was efficient in wound healing, or as an agent for re-epithelization was therefore evaluated.

These multiform aspects of chitosan parallel those as a drug carrier, and make this polymer unique in the pharmaceutical field, and indicate that its potential applications may be even wider than those currently examined and reported to date. ***(Ravi Kumar, M.N.V., 2000; Kumar, M.N., 2001; Gupta, K.C., 2000).***

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