CHAPTER I

INTRODUCTION

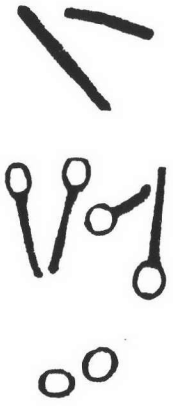
Tetanus (William, 1968)

This disease has been known for centuries. It is characterized by strong and continuous contractions of the voluntary muscles, beginning often in the neighborhood of an infected wound, usually involving all the muscles eventually and causing death. The frequency with which the muscles of the jaw and neck are contracted early in the disease has given rise to popular term "lockjaw". Although tetanus is commonly thought of as a dangerous complication of war wounds, it occurs not infrequently in civil life. Tetanus bacillus was first described in 1884 by Nicolaier, who observed it in the pus taken from mice and other animals that had died after subcutaneous inoculation with small quantities of soil. Kitasato isolated the microorganism in pure culture in 1889 and established its etiologic relation to the disease. He also showed the inability of the tetanus bacillus to invade the blood stream and showed the disease to be an intoxication. In 1890 von Behring and Kitasato laid the basis for antitoxic therapy in their discovery of diphtheria and tetanus antitoxins.

Clostridium tetani spores exist in many soils, especially if they are manured, and the organism is sometimes found in the lower intestinal tract of humans and animals.

Clostridium tetani. Morphology and Staining. The tetanus organism is a slender, Gram-positive bacillus. Young forms are motile. It develops round spores, which are

larger in diameter than the rod and appear at the very end, so that the bacterium looks like a microscopic drumstick.



Vegetative forms

Drumstick-shaped sporulating forms

Free spores

Occurrence. The tetanus bacillus is a common inhabitant of the intestine of horses and other herbivorous animals, and also it is frequently present in the human intestine. This very dangerous organism can exist there without harm because its toxin is destroyed by proteolytic enzymes of the digestive tract. It forms spores when it leaves the body in the feces and is deposited upon the soil. The tetanus spores are extremely resistant. If protected from sunlight or other injurious influences, they remain alive for many years. These resistant spores become widely distributed in the soil and on dirty objects everywhere.

Physiological Properties ; Tetanus toxin. The tetanus bacillus is a strict anaerobe. Surface growth can be secured in the laboratory only by use of an efficient anaerobic jar, or some other method for removing contact with atmospheric oxygen. It will grow, however, in cooked-meat medium and in plain liquid or semisolid media containing thioglycollate. Growth is abundant on enriched media, such as blood agar, at body temperature. Agar surface colonies are rhizoid ; that is, they have filamentous, root like outgrowths. This results from the habit of forming long, curling chains. The organism is readily distinguishable from other principal members

of the Clostridium genus by its distinctive "drumstick" sporulating form and colony, and by its failure to produce fermentation of carbohydrates or other common biochemical changes.

The outstanding property of the tetanus bacillus is its capacity to produce a powerful, specific exotoxin. The toxin is a simple protein, having a molecular weight of 67,000. It has a peculiar affinity for nervous tissue, including the peripheral nerves, and especially for the motor nerve centers in the central nervous system. When the poison reaches these centers, tetanic convulsions of the muscles follow.

The tetanus toxin is one of the most powerful poisons known. In purified form 1 mg may contain enough toxin to kill 35 million mice. Human beings are as susceptible to this toxin as mice. It is easy to understand, therefore, how a slight development of the tetanus organisms in a wound may be sufficient to cause a fatal toxemia.

Like diphtheria toxin, tetanus is inactivated by heat (five minutes at 65°C) and is converted to a harmless toxoid by treatment with formalin.

Tetanus in Man. Special Features of this Infection. Despite the almost universal distribution of tetanus spores, and the frequent occurrence of wounds which must be contaminated with them, tetanus is not a common disease. This is explained by the fact that the tetanus spores cannot germinate and multiply to produce toxin unless conditions in the wound suit their requirements. They are saprophytes, and not adapted to invasion of healthy tissue ; hence the presence of some dead flesh, which may give them a start, is probably essential. The presence of other organisms

in the wound favors the development of the tetanus germs. Further, the tetanus spores will not germinate and multiply unless carried deep into the tissues, where there is little atmospheric oxygen, or unless accompanied by aerobic organisms that use up the oxygen. Hence badly lacerated wounds in which splintered wood or bits of glass are embedded, gunshot wounds, and wounds made by blank cartridges in which particles of paper or cottonwadding bearing tetanus spores are driven into the flesh, and other types of penetrating wounds, are especially liable to furnish the proper conditions for growth of tetanus bacilli.

Rarely, tetanus has occurred following contamination of a smallpox vaccination sore, or other superficial wound or ulceration, and after surgical operations upon the intestine. The use of suture material contaminated with tetanus spores probably explains most of these rare cases of postoperative tetanus. In infants, fatal tetanus may follow if the germs enter the cut surface of the umblilical cord.

In any case, the tetanus bacilli multiply in the wound to a limited extent only; they do not invade the deeper tissues. The disease is caused entirely by absorption of the powerful tetanus neurotoxin by the motor nerve trunks. It travel up the spinal cord to the central nervous system. The incubation period, in the usual cases of severe generalized tetanus, is about seven days. The tonic contractions of the muscles, which often begin in the region of the wound and early affect the muscles of the jaw, finally extend to the whole body, causing paralysis of respiration and death. The clinical features of tetanus are usually so clear-cut that they permit early diagnosis without laboratory aid.

Microcapsules

Microcapsules developed for use in medicine, consist of a solid or liquid core material, containing one or more drugs enclosed in coating (Patrick,1984). The capsule wall is inert to the substance it contains, possesses enough strength to allow for normal handling without rupture, and is sufficiently thin to permit a high core volume to wall volume ratio. The contents of the capsule are contained within the wall until released by some means that serve to break, crush, melt, dissolve, rupture or remove the capsule shell, or until the internal phase is caused to diffuse out through the capsule wall.

With the use of microencapsulated compositions, handling problems are facilitated. Materials which would react with one another on contact, can be individually encapsulated and then mixed without premature reaction. Liquid-filled microcapsules have low vapor pressure thereby eliminating any toxicity hazard during handling. Microcapsules can be uniformly distributed on a matrix body or coating. They can also be used to fill macrocapsules. Since encapsulated fills are protected from air, moisture, microorganisms and other contaminants, spoilage is reduced and shelf life increased (Gutcho, 1976).

Microcapsules usually have a particle size range between 1 and 2000 microns.

Products smaller than 1 micron are referred to as nanocapsules, because their dimensions are measured in nanometers (Patrick, 1984).

The name of microcapsules are always changed as their composition of the membrane ; phospholipids called "liposome" ; surfactants called "neosome" etc. (Prescott, and Nimmo, 1990).

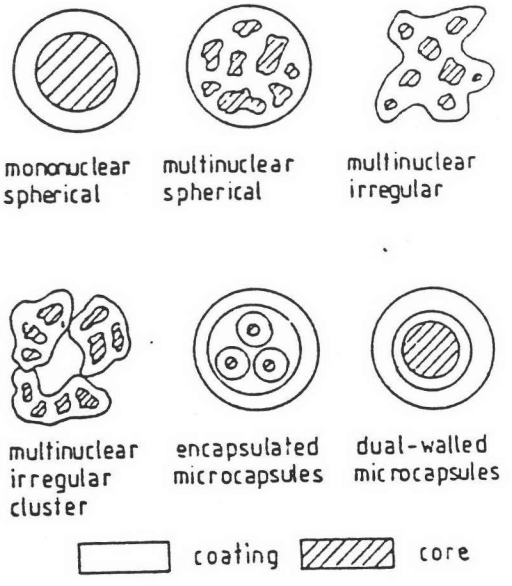


Figure 1 Some typical structure of microcapsules

Major Elements of Microcapsules Systems (Herman, 1970)

1) Core

The core phase, and consequently the capsule itself, may represent a wide range of possible configurations. Some of these are shown in Fig. 2. Operations that may be required in preparation of suitable cores include : Spheroidization, prilling, emulsification, grinding, and atomization. Selection of the preferred technique of core preparation may be of considerable importance as the configuration of the resulting capsule may strongly influence the performance of the capsular product in the end use application.

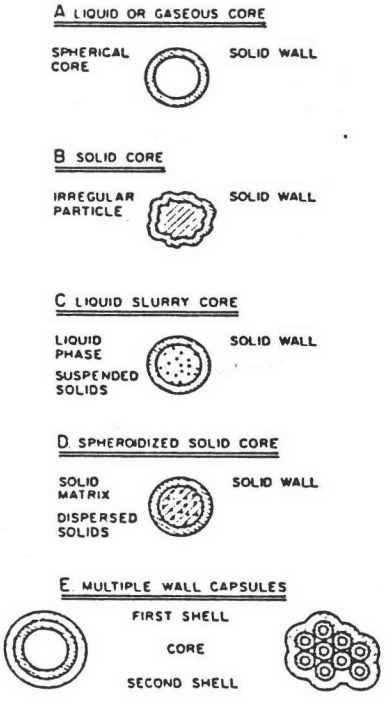


Figure 2 Capsule configurations

2) Wall

The selection of the proper wall material or materials for a given product application is often determined by the requirements of the system under consideration. For example, if the capsular product must resist the leaching action of an aqueous medium, the wall material selected should be hydrophobic in nature and provide a good water barrier. Since the wall deposited tends to follow the contour of the core (as indicated in Fig. 2), the final form of the capsular product is affected by the core configuration. Depending upon the encapsulation process employed, it may be more or less difficult to coat uniformly core particles whose surfaces posses sharp peaks and depression much like the problems encountered in conventional painting or plating.

3) Microencapsulation Process Selection

The selection of the preferred microencapsulation process for a given application is not always a simple task for there are a large number of processes from which to choose. One must be concerned with such factor as : whether the core is solid or liquid ; the solubility characteristics of the core ; the reactivity of the core with candidate wall materials and solvents ; the size of the desired capsule ; the method of attaching the capsule to the desired substrate ; the method of core release ; and process and product economics.

4) Microcapsules Post-Treatment

In many instances the microcapsules made by a given process require additional treatment to impact the desired properties. Post-treatment may involve chemical and/or physical methods. Hardening of gelatin walls by treatment with formaldehyde is employed in microcapsules used for carbonless carbon paper. Polymer wall films deposited from organic solvent solutions may be hardened by treatment with a selected nonsolvent for the wall material. Heat-fusible wall materials deposited on core particles may be improved in barrier properties by a suitable heat treatment. Thin coatings of liquids and finely divided solids may be applied to the microcapsules wall surface to reduce the tendency of microcapsules to adhere to each other and to improve barrier properties in selected enviroments.

5) Microcapsules-Substrate Interaction

Although there are instances where microcapsules, as produced, are used directly in a product, in numerous cases the microcapsules must be fastened to a substrate or suspended in a medium for proper functioning in the end use. For example, food flavor microcapsules may be suspended in a matrix of jelly in a tart and must remain stable until release is effected by heating in the toaster.

Here two problems are encountered : the need for a method of applying microcapsules to substrate and the need to avoid adverse effects of substrate on microcapsules stability in storage. The method selected for applying the microcapsules to the substrate must avoid the use of solvents that tend to leach core material through the microcapsules wall during the application period. In addition, the

presence of the binder employed to fasten microcapsules to substrate should not significantly interfere with subsequent capsular release. Products which require suspension of microcapsules in a matrix must be designed so that the microcapsules contents and wall remain intact during the time the product is stored prior to consumption. If the microcapsules are surrounded by an aqueous medium the wall material must limit the rate of leaching of core so that sufficient active core material remains at the time release is desired.

6) Storage

Conditions under which microcapsules and products containing microcapsules are stored vary widely. Factors of concern include temperature, humidity, pressure, light, or other forms of radiation and air polluants. Microcapsules containing volatile materials or certain reactive chemicals must be protected from excess temperature to avoid premature evaporation or decomposition of the core contents. If the core is hygroscopic, microcapsules may absorb water from a high humidity atmosphere to the point of wall rupture, in some cases. Conversely, microcapsules containing some water in the core may lose water by evaporation in a low humidity environment, thereby reducing the reactivity of the capsular system. Excessive pressure on microcapsules in storage, such as certain slow release fertilizer, may cause blocking or welding together of the microcapsules so that the product is no longer free-flowing. Stacking of microcapsules containing products may cause premature rupture of microcapsules in the bottom layer of the stack.

7) Release and Reaction

Capsular core release may be instigated by such methods as : pressure and shear to rupture the wall, heat to melt wall material, dissolution of the wall by a solvent, and extraction of the core contents by leaching through the wall. The mechanism selected is governed by the end use application of the product.

Release of core contents is usually followed by some chemical reaction with a material adjacent to the capsule, or a physical effect such as evaporation or wetting. The efficiency of release and mixing with a co-reactant is important to minimize the quantity of microcapsules required, hence cost. If speed of reaction is important, release should involve initimate contact with a co-reactant and avoid losses of core material to substrate of surroundings.

Reasons For Microencapsulation (Patrick, 1984)

There are many reasons why drugs and related chemicals have been microencapsulated.

Microencapsulation has been employed to provide protection to the core material against atmospheric effects. Vitamin A palmitate microcapsule had enhanced stability compared to the unencapsulated control. The process has been used to reduce the volatility of several substance such as methyl salicylate and peppermint oil. Also, incompatibilities between drugs such as aspirin and chlorpheniramine maleate can be prevented by microencapsulation (Bakan and Anderson, 1976). Pharmaceutical eutetics have been microencapsulated to effect separation.

Many drugs have been microencapsulated to reduce gastric and other gastrointestinal tract irritation, including ferrous sulfate (Elwood and Williams, 1970) and potassium chloride (Arnold, Jacob, and Riley, 1980). The local irritation and release properties of a number of topically applied products can be altered by microencapsulation (Sudekum, 1976). Liquid crystals have been microencapsulated for use in thermography of peripheral vascular disorders (Maggi and Roberto, 1976) Microcapsules have also been proposed as an intrauterine contraceptive device (Gardner, Fink, and Hassler, 1981).

Other characteristics, it has an adjuvant effect in the formation of antibodies i.e, tetanus toxoid (Davis and Gregoriadis, 1987).

However, the use of microencapsulation for the production of sustained-release dosage forms has been widely employed in the last 30 years. These dosage

forms are often described by other terms, such as delayed-action, long-acting, retard, slow-release, sustained-action, or timed-release. Sometimes the term "Controlled release" is used in the same sense or where, for example, an enteric coating is applied to localize the core release in the small intestine rather than the stomach, sustained release of entrapped drugs i.e, interferon and a peptide hormone (calcitonin) (Weiner, Martin and Riax, 1989), diphtheria toxoid (Singh, M., Singh, A., and Talwar, 1991).

Microencapsulation Techniques

1. Physicochemical methods i.e, aqueous and organic phase separations and spray-drying method.

2. Chemical method i.e, interfacial polymerization, in situ polymerization, and orifice method.

3. Physical method i.e, physical vapor deposition, electrostatic, and fluidized-bed spray-coating methods.

In this research one use the interfacial deposition method.

Interfacial Polymerization Method (Patrick, 1984)

Interfacial polymerization involves the reaction of various monomers at the interface between two immiscible liquid phases to form a film of polymer that encapsulates the disperse phase. Usually two reactive monomers are employed, one dissolved in the aqueous disperse phase containing a solution or dispersion of the core material, and the other dissolved after the emulsification step in the nonaqueous continuous phase. The water-in-oil (w/o) emulsion formed requires the addition of a suitable emulgent as stabilizer.

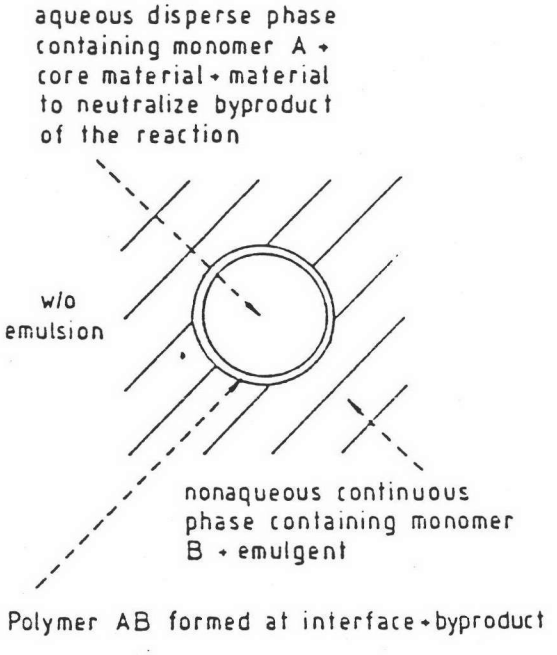


Figure 3 Schematic representation of microencapsulation of a droplet by interfacial polymerization.

This process, the monomers diffuse together and rapidly polymerize at the interface between the phase to form a thin coating, and the byproduct of the reaction is neutralized by added material such as an alkaline buffer. The degree of polymerization can be controlled by the reactivity of the monomers chosen, their concentration, the composition of either phase vehicle, and by the temperature of the system. Variation in particle size of the disperse phase controls the particle size of the product. The reaction between the monomers is quenched by depletion of monomer, which is frequently accomplished by adding excess continuous-phase vehicle to the emulsion.

The interfacial polymerization method is suitable for encapsulating liquids rather than a solid phase. The following steps of interfacial polymerization are :

1. Dispersion of an aqueous solution of a water-soluble reactant or an organic solution of an oil-soluble reactant into an organic or an aqueous phase with the aid of an appropriate emulsifier to yield a water-in-oil emulsion or an oil-in-water emulsion.

2. Formation of polymer membrane on the surface of liquid droplets initiated by the addition of a water-insoluble reactant to the water-in-oil emulsion or a water-soluble reactant to the oil-in-water emulsion.

3. Separation of microcapsules containing water or oil from the oil or aqueous phase.

Tetanus Toxoid Microcapsules Using Lecithin as Polymeric Membrane and Carboxymethylchitin as Stabilizer (Natesuwon, 1992)

Using microcapsules for human, the biophysical and biochemical properties had been considered in comparison with those of natural mammalian cells because it is feared that the chemicals used in the preparation may be trapped in the human body or may cause immunological reaction before they are degraded in the body. Thus, the use of such non toxic, biocompatible, biodegradable and non antigenic substance as lecithin, one type of phospholipid, is required to prepare microcapsules as polymeric membrane and carboxymethylchitin as stabilizer.

As the interfacial polymerization technique, two steps of emulsification were required for preparation of these microcapsules. Tetanus toxoid was dispersed as fine droplets in a lecithin solution in dichloromethane, a volatile organic solvent to yield a w/o type emulsion. Then, this w/o emulsion was dispersed in an aqueous carboxymethylchitin solution to give a w/o/w type complex emulsion. Removal of the organic solvent by evaporation from the complex left an aqueous suspension of the tetanus toxoid microcapsules.

Tetanus Toxoid, Tetanus Toxoid Adsorbed

1. Manufacturing

Production of a typical tetanus toxoid begins with the growth of a highyielding strain of Clostridium tetani in a liquid, nonantigenic medium. After a suitable period, the culture supernatant fluid (extracellular toxin) and/or cells (intracellular toxin) are harvested and are usually purified further, often by alcohol or salt fractionation. Most manufacturers detoxify (toxoid) the crude toxin before purification, in part for the safety of production personnel.

The toxoiding process involves treatments of the toxin with a dilute solution of formaldehyde for a period of days or weeks, during which time the product may be tested for toxicity in animals. The product is labeled as tetanus toxoid only when it no longer exhibits any signs of tetanus toxicity. When the bulk toxoid has been prepared, it may be mixed with other antigens as in the case of DTP (diphtheria, tetanus, pertussis), for example and/or combined with an insoluble, inorganic salt (e.g., aluminium phosphate) to enhance its immunogenicity. Numerous variations in the process outlined here are used commercially and result in acceptable vaccines. The WHO has prepared a detailed manual for the production and control of tetanus toxoid (WHO, 1977). Common requirments include assessment of sterility, potency in animal test, purity, freedom from toxicity, freedom from reversion to toxicity and product stability.

Chemistry and Stability (Gerald, 1992)

Chemistry

Tetanus toxoid is a sterile suspension of the formaldehyde-treated products of growth of Clostridium tetani. Tetanus toxoid adsorbed is tetanus toxoid which has been adsorbed onto aluminum hydroxide, aluminum phosphate, or potassium alum. Each 0.5 ml of tetanus toxoid adsorbed contains not more than 0.85 mg of aluminum when determined analytically, or not more than 1.14 mg when calculated on the basis of the amount of aluminum compound added. Both preparations meet standards established by the office of Biologics of the US Food and Drug Administration. The antigen content of the toxoids is expressed in flocculating units (Lf). Depending on the manufacturer, each 0.5 ml of commercially available tetanus toxoid contains 4 or 5 Lf units of tetanus toxoid and each 0.5 ml of commercially available tetanus toxoid adsorbed contain 5 or 10 Lf units of tetanus toxoid.

Commercially available tetanus toxoid is a clear, colorless to brownish yellow, or slightly turbid liquid which may have a characteristic odor or an odor of formaldehyde. Commercially available tetanus toxoid adsorbed is a turbid and white, slightly gray, or slightly pink suspension. The toxoids contain thimerosal as a preservative and made isotonic with sodium chloride.

Stability

Tetanus toxoid and tetanus toxoid adsorbed should be refrigerated at 2-8°C and should not be frozen. The expiration date for tetanus toxoid or tetanus toxoid adsorbed is not later than 2 years after the date of issue from the manufacturer's cold storage (e.g., 1 year when the manufacturer's cold storage was 5°C). The toxoids

should be free from clumps after vigorous shaking and should not be used if resuspension cannot be achieved.

Pharmacology

Tetanus toxoid and tetanus toxoid adsorbed promote active immunity to tetanus by inducing production of specific antitoxin. A single IM dose of either toxoid does not provide protection against the disease. However, completed primary immunization with tetanus toxoid adsorbed or tetanus toxoid in susceptible individuals induces production of antitoxin levels which result in immunity to tetanus that persists for at least 10 years in most individual. Although the rate of seroconversion is essentially the same with either form of the toxoid, tetanus toxoid adsorbed induces more persistent antitoxin titers than tetanus toxoid.

Uses

Tetanus toxoid and tetanus toxoid adsorbed are used to provide active immunity to tetanus in adults and children older than 6 weeks of age. The US Public Health Service Immunization Practices Advisory Committee (ACIP) and the Committee on Infectious Diseases of the American Academy of Pediatrics (AAP) currently recommend that all individuals receive immunization against both diphtheria and tetanus, and that children younger than 7 years of age also receive immunization against pertussis. Therefore, diphtheria and tetanus toxoid and pertussis vaccine adsorbed (DTP) is the preparation of choice for primary and booster immunization against tetanus in children 6 weeks through 6 years of age and tetanus and diphtheria toxoids adsorbed (Td) is the preparation of choice for primary and booster immunization against tetanus in individuals 7 years of age or older. The ACIP states that because adults are generally less likely to have adequate circulating levels of

diphtheria antitoxin than adequate circulating levels of tetanus antitoxin, Td should be used routinely in all medical setting for individuals 7 years of age or older requiring primary immunization or booster doses of tetanus toxoid adsorbed. The proportions of individuals lacking protective levels of circulating antitoxin against diphtheria and/or tetanus increase with age ; at least 40% of individuals 60 years of age or older may lack protection. Tetanus toxoid or tetanus toxoid adsorbed should be used for primary or booster immunization against tetanus only when preparations containing combined antigens are contraindicated. Because tetanus toxoid adsorbed induces more persistent antitoxin titers than tetanus toxoid, it is the preferred preparation when the individual tetanus antigen is indicated. Tetanus infection does not necessarily confer immunity ; therefore, initiation or completion of primary immunization is indicated at the time of recovery from this infection.

Spores of Clostridium tetani are ubiquitous and there is essentially no natural immunity against tetanus toxin. Universal, primary immunization and maintenance of adequate antitoxin levels with appropriately timed booster dose is necessary for all ages. To maintain adequate immunity against tetanus following primary immunization with tetanus toxoid or tetanus toxoid adsorbed it is necessary to administer routine booster doses of the toxoid every 10 years. For individuals who are going to summer camps or on wilderness expeditions where tetanus toxoid or tetanus toxoid adsorbed may not be readily available, some clinicians recommend that administration of a booster dose of the toxoid be considered if more than 5 years have elapsed since primary or booster immunization against tetanus. In the event of injury and possible exposure to tetanus infection, the need for active immunization with or without passive immunization, depend on the individual's history of tetanus immunizations and the likelihood of contamination with tetanus bacilli (e.g., condition of the wound,

source of contamination). The ACIP currently describes wounds as being tetanus prone or as clean, minor wounds. The ACIP states that tetanus-prone wound include, but are not limited to, wounds contaminated with dirt, feces, soils or saliva ; puncture wounds ; avulsion and wounds resulting from missiles, crushing, burns, or frostbite. Alternatively, some clinicians describe wounds as being very tetanus prone, moderately tetanus prone, or not tetanus prone. A wound is considered very tetanus prone if it has been exposed to a high level of bacterial contamination is more than 24 hours old at the time of treatment, or contains devitalized tissue that cannot be completely debrided. A wound is considered moderately tetanus prone if it has been exposed to a moderate level of bacterial contamination is a crush injury or puncture wound, or extends into muscle. Although wounds from human bites are usually highly contaminated, they are generally considered moderately tetanus prone, since tetanus is not part of the normal oral flora. A wound is considered not tetanus prone if it is 24 or less hour old, has a low level of bacterial contamination, contains no devitalized tissue, is not a crush injury or puncture wound, and does not extend into muscle. Neonatal tetanus occurs in infants born under conditions where infection is likely to women who are not adequately immunized ; immune pregnant women confer protection to their infants through transplacental maternal antibody.

Administration

Tetanus toxoid is administered by intramuscular or subcutaneous injection and tetanus toxoid adsorbed is administered only by deep intramuscular injection, preferably into the midlateral muscles of the thigh or deltoid. The same muscle site should not be used more than once during the course of primary immunization. Tetanus toxoid and tetanus toxoid adsorbed should be inspected visually for particulate matter and discoloration prior to administration. To ensure a uniform suspension

of antigen, containers of tetanus toxoid adsorbed should be shaken vigorously prior to withdrawing a dose.

Dosage

Tetanus toxoid and tetanus toxoid adsorbed are administered in 0.5 ml doses. Depending on the manufacturer, each 0.5 ml dose of tetanus toxoid contains 4 or 5 Lf units of tetanus toxoid and each 0.5 ml of tetanus toxoid adsorbed contains 5 or 10 Lf units of tetanus toxoid.

For primary immunization in adults or children 6 weeks of age or older, some manufacturers suggest that tetanus toxoid adsorbed is given in a series of 3 doses ; the second dose is given 4-8 weeks after the first dose, and the third dose is given 6-12 months after the second dose. However, the ACIP and AAP currently recommend that children 6 weeks through 6 years of age receive 4 doses of tetanus toxoid adsorbed for primary immunization ; the first 3 doses are given 4-8 weeks apart, usually at 2, 4 and 6 months of age, and the fourth dose is given approximately 6-12 months after the third dose (usually at 15-18 months of age). The ACIP and AAP also recommend that children who received the fourth dose of tetanus toxoid adsorbed before their fourth birthday receive a fifth dose at 4-6 years of age (usually just prior to entry into kindergarten or elementary school). For primary immunization in adults and children 7 years of age or older, the ACIP and APP currently recommend that tetanus toxoid adsorbed is given in a series of 3 doses ; the second dose usually is given 4-8 weeks after the first dose, and the third dose is given 6-12 month after the second dose. If tetanus toxoid is used for primary immunization, the manufacturers recommend that 3 doses are given 3-8 weeks apart and a fourth dose is given 6-12 months after the third dose. Interruption of the recommended

immunization schedules, regardless of the length of time between doses, does not interfere with the final immunity achieved, nor does it necessitate additional doses or starting the series over.

2. Efficacy of Tetanus Toxoid

The efficacy of tetanus toxoid is evaluated by potency testing and antibody titer determination.

2.1 Potency Testing (WHO, 1990)

Each final bulk of tetanus toxoid will be tested for immunizing potency by comparison with a national reference material calibrated against the appropriate international standard. The test will involve the inoculation of groups of guinea-pigs (weight 250-350 gm) or mice (weight 14-20 gm provided that, in a single test, the individual weights of the mice will not vary by more than 3 gm). Three dilution of both the final bulk and reference material will be used. After immunization, the animals will be challenged with a lethal or paralytic challenge dose of toxin given by the subcutaneous route. Standard statistical method will be used to calculate the potency of the final bulk. The method adopted and its interpretation will be approved by the national control authority.

2.2 Antibody Titer Determination by Enzyme - Linked Immunosorbent Assay (ELISA)

In the early 1970_s, the search for simple sensitive methods for detecting and quantitating antigen and antibody that did not rely upon particle agglutination or radiolabelled reagents led to the development of solid phase enzyme -

coupled reagent assay. In principle, the labelling by chemical conjugation of an enzyme to either antigen or antibody allows detection of immune complexes formed on a solid phase, as the fixed enzyme, once washed free of excess reagents, on subsequent substrate interaction can yield a coloured product which can be visualized and / or measured by optical density (Catty, 1990).

Immunoassay has been applied to determine hormones, enzymes, proteins, drugs, and infectious agents. Solid phase enzyme immunoassays can be used reliably for large number screening of small volume test sample in the simplest of laboratory environments. Immunoassays especially ELISA is a method which has a high degree of sensitivity and reproducibility and it is well suited for routine use and is reasonably inexpensive (Simonsen, Bentzon, and Heron, 1986).

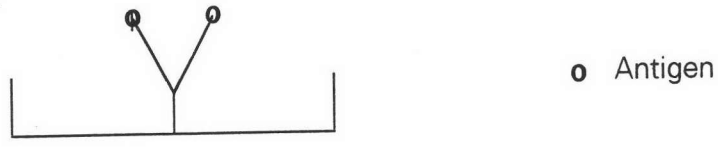
ELISA, the covalent attachment of enzymes to antibody molecules creates an immunological tool possessing both high specificity and high sensitivity. ELISA makes use of antibodies to which enzymes have been covalently bound such that the enzyme's catalytic properties and the antibody's recognition properties are retained. Typical liked enzymes include peroxidase, alkaline phosphatase, and β-galactosidase, all of which catalyze reactions whose products are colored and can be measured in very low amounts.

Two basic ELISA methodologies have been developed, one for detecting antigen (direct ELISA) and the other for detecting antigens or antibodies (indirect ELISA). For detecting antigen by a direct ELISA, such as detecting virus particles from a blood or feces sample, the so-called double-antibody sandwich technique is used. In this procedure the antigen is "trapped" (sandwiched) between two layers of

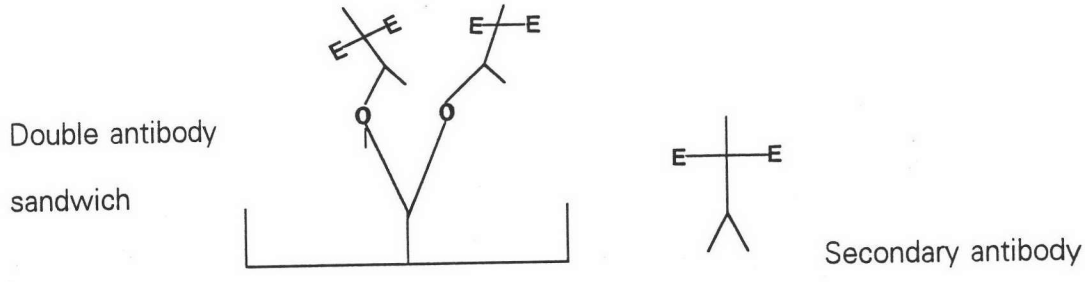
antibodies. The specimen is added to the wells of a microtiter plate previously coated with antibodies specific for the antigen to be detected. If the antigen is present in the sample it will be trapped by the antigen binding sites on the antibodies. After washing unbound material away a second antibody containing a conjugated enzyme is added. The second antibody is also specific for the antigen so it will bind to any remaining exposed determinants. Following a wash, the enzyme activity of the bound material in each microtiter well is determined by adding the substrate of the enzyme. The color formed is proportional to the amount of antigen originally present.

To detect antibodies in human serum, an indirect ELISA is used. The microtiter wells in this case are coated with antigen and a serum sample added. If antibodies to the antigen are present in the serum, they will bind to the antigen in the wells. Following a wash, a second antibody is added. The second antibody is a rabbit or goat anti-human IgG antibody preparation containing a conjugated enzyme (this antiserum is prepared by injecting a goat or a rabbit with human IgGs ; IgGs these will be recognized as foreign and goat or rabbit anti-human IgG antibodies are produced). Following the addition of enzyme substrate a color is formed, and the amount of circulating human antibody to the specific antigen is quantitated from the intensity of the color reaction. (Thomas and Michael, 1988) Diagram of Direct ELISA and Indirect ELISA are shown in figure 4, 5.

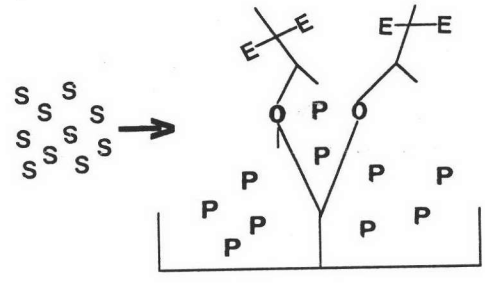
1. Antibody to antigen is bound to microtiter well



2. Blood sample added as source of antigen

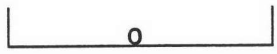


3. Antibody labeled with enzyme (E) added.
Enzyme-labeled antibody binds to antigen



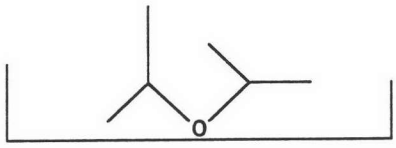
4. Substrate (S) for enzyme is added. Colored product of enzyme formed (P) ; color is proportional to amount of antigen

Figure 4 Direct ELISA for detecting antigen (double antibody sandwich method).



o Antigen

1. Antigen is bound to microtiter well

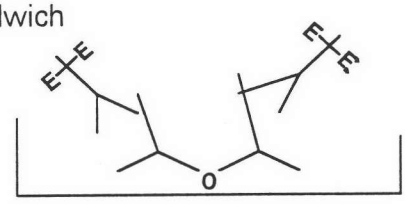


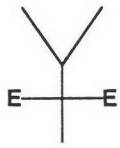
Antibody from serum

2. Antibody from serum added ; wash

Open-faced

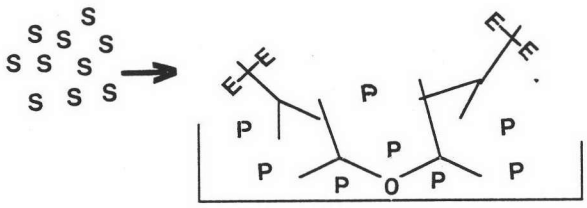
sandwich





Secondary antibody

3. Anti-human immunoglobulin antibody labeled with enzyme (E) added ; wash



4. Add enzyme substrate (S). Color formed (P) is proportional to amount of antibody in serum

Figure 5 Indirect ELISA for detecting specific antibodies (open-faced sandwich method).

So, the performance of ELISA depends on four major principles.

I) A variety of enzymes, including horseradish peroxidase (HRP) and alkaline phosphatase (AP) - the most popular can be chemically coupled to either antibody or antigen under conditions which retain the biological properties (i.e.substrate interaction, antigen binding) of both components of the conjugate.

II) Most antigens, for example proteins, peptides, polysaccharides and drugs, bind spontaneously to plastic surfaces such as the wells of polystyrene microtitre plates. Antibodies, as proteins, also attach whilst retaining their antigen-binding activity. Thus antigen or antibody - coated plates can be prepared as the initial step. Once antigens or antibodies applied to coat or "sensitize" the solid phase are bound, they become resistant to vigorous washing in detergent buffer whilst excess unbound reagent is simply removed by this process.

III) In subsequent steps one or more layers of a solid phase captured immune complex are formed, with unbound entities again efficiently washed away. This affords the basis for high specific to non-specific signal ratio when captured enzyme reacts with substrate.

IV) An enzyme conjugate of antibody or antigen when bound in the immune complex leaves the enzyme component available for substrate interaction. Addition of substrate, in the usual form of assay, results in a progressive substrate solution colour change. The reaction can be stopped at an appropriate stage and the colour signal determined by visual comparison with standards or by optical density measurement.

Purpose of the study

According to the previous thesis ; "Immune response in mice produced by lecithin and carboxymethylchitin walled tetanus toxoid microcapsules" was studied. Tetanus toxoid micropsules were prepared by modified interfacial polymerization technique using purified egg yolk lecithin and carboxymethylchitin as the wall materials in order to give an effective, long acting tetanus toxoid. It can reduce the frequency of immunization by using only a single dose instead of triple dose at 2, 4 and 6 months. For further investigation the purpose of this study was the following :

1. Testing the stability of tetanus toxoid microcapsules.
2. Evaluate the immune response of tetanus toxoid microcapsules preparation by enzyme-linked immunosorbent assay and potency test.

Application for the study

To complete the study of developing long acting tetanus toxoid microcapsules. The technique used for preparation of microcapsules was approved to determine the efficiency and the stability of the product.