CHAPTER II

LITERATURE REVIEWS

I. Diphtheria

Diphtheria is a bacterial infection caused by Corynebacterium diphtheriae (C. diphtheriae), transmitted from person to person through close physical and respiratory contact. It can cause infection of the throat which may lead to obstruction of the breathing and death. Like other respiratory infections, transmission is increased inover-crowded and poor socio-economic conditions. In temperate climates, prior to vaccination, respiratory diphtheria commonly affected preschool and school-age children, and deaths occurred from exotoxin-induced damage to other organs such as the heart. Large epidemics occurred in Europe during and after the Second World War, with an estimated one million cases and 50 000 deaths in 1943. Nasal diphtheria may be mild and chronic carriage of the organism frequently occurs; asymptomatic infections are common. A cutaneous form of diphtheria is common in tropical countries, and may be important in transmission. Recently, large epidemics have occurred in Russia and the Newly Independent States (NIS).

1. Clinical description

The incubation period of diphtheria is 2–5 days (range, 1–10 days). Disease can involve almost any mucous membrane. For clinical purposes, it is convenient to classify diphtheria into a number of manifestations, depending on the site of disease.

Anterior nasal diphtheria: the onset is indistinguishable from that of the common cold and is usually characterized by a mucopurulent nasal discharge (containing both mucus and pus) which may become blood-tinged. A white membrane usually forms on the nasal septum. The disease is usually fairly mild because of apparent poor systemic absorption of toxin in this location, and can be terminated rapidly by antitoxin and antibiotic therapy.

Pharyngeal and tonsillar diphtheria: the most common sites of infection are the pharynx and the tonsils. Infection at these sites is usually associated with substantial systemic absorption of toxin. The onset of pharyngitis is insidious. Early symptoms include malaise, sore throat, anorexia, and lowgrade fever. Within 2–3 days, a bluish-white membrane forms and extends, varying in size from covering a small patch on the tonsils to covering most of the soft palate. Often by the time the person seeks medical attention, the membrane is greyish-green in color, or black if there has been bleeding. There is a minimal amount of mucosal erythema surrounding the membrane. The membrane is adherent to the tissue, and forcible attempts to remove it cause bleeding. Extensive membrane formation may result in respiratory obstruction. The patient may recover at this point; or if enough toxin is absorbed, develop severe prostration, pallor, rapid pulse, stupor, coma, and may die within 6 to 10 days. Fever is usually not high, even though the patient may appear quite toxic. Patients with severe disease may develop marked edema of the submandibular areas and the anterior neck along with lymphadenopathy, giving a characteristic "bullneck" appearance.

Laryngeal diphtheria: Laryngeal diphtheria can be either an extension of the pharyngeal form or the only site involved. Symptoms include fever, hoarseness, and a barking cough. The membrane can lead to airway obstruction, coma, and death.

Cutaneous (skin) diphtheria: Skin infections are quite common in the tropics and are probably responsible for the high levels of natural immunity found in these populations. Skin infections may be manifested by a scaling rash or by ulcers with clearly demarcated edges and membrane, but any chronic skin lesion may harbor C. diphtheriae, along with other organisms.

Most complications of diphtheria, including death, are attributable to effects of the toxin. The severity of the disease and complications are generally related to the extent of local disease. The toxin, when absorbed, affects organs and tissues distant from the site of invasion. The most frequent complications of diphtheria are myocarditis and neuritis.

2. Bacteriology and pathogenesis

Corynebacterium diphtheriae is an aerobic gram-positive bacillus.Toxin production (toxigenicity) occurs only when the bacillus is itself infected (lysogenized) by a specific virus (bacteriophage) carrying the genetic information for the toxin (tox gene). Only toxigenic strains can cause severe disease. Culture of the organism requires selective media containing tellurite. If isolated, the organism must be distinguished in the laboratory from other Corynebacterium species that normally inhabit the nasopharynx and skin (e.g., diphtheroids). There are four biotypes of C. diphtheriae (gravis, mitis, belfanti, and intermedius), which historically were identified by colonial morphology and biochemical differences; however, in practice, only the intermedius biotype can be distinguished reliably by colonial morphology. No consistent differences are found in severity of disease caused by different biotypes.

Identified features of C. diphtheriae that are important in the pathogenesis of the disease in humans comprise certain cell wall antigens and in particular the organism's exotoxin. 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 and therefore permit categorization of the organisminto a number of types. 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.

3. Diphtheria toxin

The exotoxin produced by C. diphtheriae is by far the most important pathogenetic factor. 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. 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.

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 (Wharton and Vitex , 2004).

4. Epidemiology

Diphtheria occurs worldwide, but clinical cases are more prevalent in temperate zones. In the United States during the pretoxoid era, the highest incidence was in the Southeast during the winter. More recently, highest incidence rates have been in states with significant populations of Native Americans. No geographic concentration of cases is currently observed in the United States. Human carriers are the reservoir for C. diphtheriae, and are usually asymptomatic. In outbreaks, high percentages of children are found to be transient carriers. Transmission is most often person-to-person spread from the respiratory tract. Rarely, transmission may occur from skin lesions or articles soiled with discharges from lesions of infected persons (fomites). In temperate areas, diphtheria most frequently occurs during winter and spring. Transmission may occur as long as virulent bacilli are present in discharges and lesions. The time is variable, but organisms usually persist 2 weeks or less, and seldom more than 4 weeks, without antibiotics. Chronic carriers may shed organisms for 6 months or more. Effective antibiotic therapy promptly terminates shedding.

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. Preschool and school-age children are most often affected by respiratory diphtheria. Diphtheria was rare in infants younger than 6 months, presumably because of the presence of maternal antibody, and rare among adults, especially those living in urban areas, as a result of

acquired immunity. Transplacental antitoxic immunity to diphtheria is present at birth in most infants but declines to nonprotective levels during the second 6 months of life.

II. 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 (Habig and Tankersley, 1990; Wassilak, Roper Murphy and Orenstein, 2004).

1. Clinical description

The incubation period ranges from 3 to 21 days, usually about 2 days. In general the further the injury site is from the central nervous system, the longer the incubation period. The shorter the incubation period, the higher the chance of death. In neonatal tetanus, symptoms usually appear from 4 to 14 days after birth, averaging about 7 days.

There is a direct relationship between the site of inoculation and the incubation period, with the longest intervals occurring after injuries farthest from the CNS; injuries of the head and trunk generally are associated with the shortest incubation periods. Incubation periods of 10 days or more tend to result in mild disease, whereas incubation periods within 7 days of injury tend to result in more severe disease (Wassilak et al., 2004).

On the basis of clinical findings, three different forms of tetanus have been described. Local tetanus is an uncommon form of the disease, in which patients have

persistent contraction of muscles in the same anatomic area as the injury. These contractions may persist for many weeks before gradually subsiding. Local tetanus may precede the onset of generalized tetanus but is generally milder. Only about 1% of cases are fatal. Cephalic tetanus is a rare form of the disease, occasionally occurring with otitis media (ear infections) in which *C. tetani* is present in the flora of the middle ear, or following injuries to the head. There is involvement of the cranial nerves, especially in the facial area. The most common type (about 80%) of reported tetanus is generalized tetanus. The disease usually presents with a descending pattern. The first sign is trismus or lockjaw, followed by stiffness of the neck, difficulty in swallowing, and rigidity of abdominal muscles. Other symptoms include elevated temperature, sweating, elevated blood pressure, and episodic rapid heart rate. Spasms may occur frequently and last for several minutes. Spasms continue for 3-4 weeks. Complete recovery may take months. Neonatal tetanus is a form of generalized tetanus that occurs in newborn infants. Neonatal tetanus occurs in infants born without protective passive immunity, because the mother is not immune. It usually occurs through infection of the unhealed umbilical stump, particularly when the stump is cut with an unsterile instrument. Neonatal tetanus is common in some developing countries (estimated more than 215,000 deaths worldwide in 1998), but very rare in the United States.

2. Bacteriology

Clostridium tetani is a gram positive, spore-forming, motile, anaerobic bacillus. Typically measuring 0.3 to 0.5 μm in width and 2 to 2.5 μm in length, the vegetative form often develops long filament-like cells in culture. 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; however, depending on the strain, growth can occur at 14 °C to 43 °C (Habig et al., 1990; Wassilak et al., 2004). The organism is sensitive to heat and cannot survive in the presence of oxygen. The spores, in contrast, are very resistant to heat and the usual antiseptics. They can survive autoclaving at 249.8 °F (121 °C) for

10-15 minutes. The spores are also relatively resistant to phenol and other chemical agents.

Sporulation is dependent on a variety of factors that include pH, temperature, and media composition. The germination of spores requires anaerobic conditions and is enhanced by the presence of lactic acid and chemicals toxic to cells (Habig et al., 1990; Wassilak et al., 2004).

The spores are widely distributed in soil and in the intestines and feces of horses, sheep, cattle, dogs, cats, rats, guinea pigs, and chickens. Manure-treated soil may contain large numbers of spores. In agricultural areas, a significant number of human adults may harbor the organism. The spores can also be found on skin surfaces and in contaminated heroin.

C. tetani produces two exotoxins, tetanolysin and tetanospasmin. The function of tetanolysin is not known with certainty. Tetanospasmin is a neurotoxin and causes the clinical manifestations of tetanus. On the basis of weight, tetanospasmin is one of the most potent toxins known. The estimated minimum human lethal dose is 2.5 nanograms per kilogram of body weight (a nanogram is one billionth of a gram), or 175 nanograms for a 70-kg (154 lb) human.

3. Pathogenesis

C. tetani usually enters the body through a wound. In the presence of anaerobic (low oxygen) conditions, the spores germinate. Toxins are produced and disseminated via blood and lymphatics. Toxins act at several sites within the central nervous system, including peripheral motor end plates, spinal cord, and brain, and in the sympathetic nervous system. The typical clinical manifestations of tetanus are caused when tetanus toxin interferes with release of neuro-transmitters, blocking inhibitor impulses. This leads to unopposed muscle contraction and spasm. Seizures may occur, and the autonomic nervous system may also be affected.

Transport of toxin from the injured site into the CNS is complex. Toxin injected under the skin appears to enter underlying muscle; infiltration of muscle with antitoxin before subcutaneous toxin injection can block the development of tetanus. Once in the muscle, some toxin makes its way to the CNS directly by intra-axonal transport; the major portion is transported by the lymphatics to the bloodstream and then disseminated to a variety of tissues (Habig et al., 1990; Wassilak et al., 2004).

4. Epidemiology

Tetanus occurs worldwide but is most frequently encountered in densely populated regions in hot, damp climates with soil rich in organic matter. Organisms are found primarily in the soil and intestinal tracts of animals and humans. Transmission is primarily by contaminated wounds (apparent and inapparent). The wound may be major or minor. In recent years, however, a higher proportion of patients had minor wounds, probably because severe wounds are more likely to be properly manages. Tetanus may follow elective surgery, burns, deep puncture wounds, crush wounds, otitis media (ear infections), dental infection, animal bites, abortion, and pregnancy.

Aside from neonatal tetanus, the largest proportion of cases in developing countries is among male older children and young adults. Wherever immunization programs are in place, the rates of tetanus decline, and the sex and age distributions shift to mirror the underimmunized population. In the 1950s, more than one third of the deaths from tetanus in the United States were among neonates and infants less than 1 year old. In contrast, from 1998 to 2000, no deaths from tetanus occurred among neonates or children, and three fourths of the deaths occurred among persons 60 years of age or older (Wassilak et al., 2004).

Tetanus toxoid is available in a plain (unadsorbed) liquid form, or adsorbed with aluminium phosphate or hydroxide, alone or in combination with other toxoids or vaccines (Clements et al., 2002).

III. Pertussis

Pertussis (whooping cough) is a bacterial infection of the nose and throat caused by *Bordetella pertussis*, a gram negative bacillus. Pertussis is a highly contagious bacterial infection that causes coughing and gagging with little or no fever. An infected person has cough episodes that may end in vomiting or cause a "whoop" sound when the person tries to breathe in. The severity of the damage which pertussis can cause to the health of young children and the frequency with which it results in death among infants less than one year old may often be underestimated. It was calculated in 1991 that, worldwide, pertussis causes the deaths of approximately 340,000 children each year. The situation is most severe in the developing countries where the case-fatality rate may exceed 1-2 percent (Granstrom, Blennow and Winberry, 1991; Locht, 1999; Edwards and Decker, 2004).

1. Clinical description

There are several detailed descriptions of the typical clinical disease in children.

Generally it can be divided into four phases: incubation, catarrhal, paroxysmal and convalescent. The causative agent, the bacterium Bordetella pertussis, is spread by aerial transmission and infection starts with its arrival in the upper respiratory tract of a susceptible host. The incubation stage involves adherence of the bacterium to the cilia of respiratory tract epithelial cells and then its multiplication, evasion of the initial non-specific immune defences of the host and colonization of the ciliated mucosa. The catarrhal stage normally lasts 7-14 days and is manifested as a mild upper respiratory tract infection, usually afebrile, with no specific symptoms. During this, the most infectious period, organisms can frequently be cultured from nasopharyngeal swabs.

A simple dry cough develops, gradually increasing in frequency and intensity. This leads into the paroxysmal stage, which usually lasts from one to six weeks and is typified by numerous sudden episodes of violent coughing. Each of these paroxysms

may consist of up to 30 rapid, forceful expiratory coughs, sometimes resulting in cyanosis. The coughing is accompanied by the expectoration of viscous mucus and often followed by vomiting. The 'whoop', which can immediately follow this characteristic coughing spasm, is due to inspiration through a narrowed glottis. It is difficult to isolate B. pertussis from the nasopharynx during the paroxysmal phase. As the disease enters the convalescent stage, the paroxysms gradually decline in frequency and severity, although a residual cough, which can be exacerbated by any intervening respiratory illness, may persist for several months.

A wide range of complications may be associated with pertussis, mainly related to the effects of the paroxysms. The violent coughing may result in umbilical or inguinal hernias, subconjunctival and, sometimes, cerebral haemorrhages. Severe anoxia during paroxysms could be the main cause of the seizures, coma, encephalopathy, permanent brain damage and deaths that are occasionally observed. Pertussis sufferers are prone to develop bronchopneumonia and are susceptible to secondary infections (Granstrom et al., 1991; Edward et al., 2004).

2. Bacteriology

The causative agent of pertussis is *B. pertussis*, a small, gram negative, pleomorphic bacillus. Two closely related organisms in the genus *Bordetella* are *B. parapertussis* and *B. bronchiseptica.* The former is responsible for a pertussis-like syndrome in humans. The latter produces respiratory illnesses in domestic animals. Of all the *Bordetella* species, only *B. pertussis* synthesizes PT (Locht, 1999).

Bordetella pertussis has a marked tropism for and attaches strongly to ciliated respiratory tract epithelial cells. The bacteria may be internalized by epithelial cells but do not penetrate submucosal cells or invade the blood stream. However, toxins produced by the organism can enter the blood stream and produce systemic effects (Granstrom et al., 1991; Edwards et al., 2004).

3. Pertussis toxin

PT, previously termed lymphocytosis promoting factor, is a major contributor to the pathogenesis of pertussis and is generally believed to play an important role in the induction of clinical immunity. PT is an oligomeric structure composed of five different subunits, Sl through S5 (Fig. 1) molecular weights; 26,024 Dalton S1, 21,924 Dalton S2, 21,873 Dalton S3, 12,058 Dalton S4 and 11,013 Dalton S5 (Locht and Keith, 1986). Structurally it belongs to the A-B class of bacterial toxins. The S1 component (A protomer) catalyzes the ADP ribosylation of GTP binding regulatory proteins involved in signal transduction in the eukaryotic cell. The A protomer is largely responsible for the recognized biologic activities of PT, including promotion of lymphocytosis, stimulation of islet cells, sensitization to histamines, clustering of Chinese hamster ovary cells, and adjuvant properties. The B oligomer is a ring shaped structure that consists of one copy each of subunits S2, S3 and S5 and two copies of S4. S5 serves to link the two dimers, S2-S4 and S3-S4. The primary function of the B oligomer is to facilitate the attachment of PT to the ciliated cells of the respiratory tract (Edwards et al., 2004).

PT appears to play two major roles in the pathogenesis of pertussis. First, it facilitates the attachment of *B. pertussis* to ciliated respiratory cells. Second, it appears to be of major importance in cell toxicity. PT is a strong immunogen.

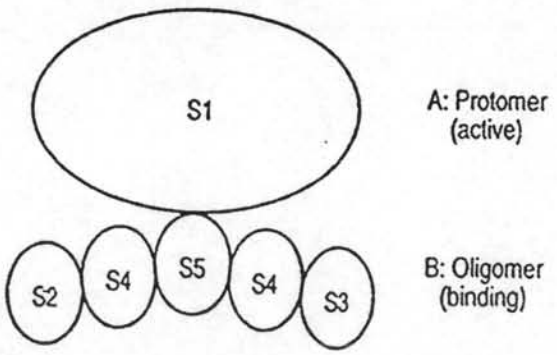

Figure 1 Diagrammatic representation of the pertussis toxin (Edwards et al., 2004)

4. Pathogenesis

Pertussis is primarily a toxin-mediated disease. The bacteria attach to the respiratory cilia produce toxins that paralyze the cilia, and cause inflammation of the respiratory tract, which interferes with the clearing of pulmonary secretions. Pertussus antigens appear to allow the organism to evade host defenses, in that lymphocytosis is promoted but chemotaxis is impaired. Until recently it was thought that *B. pertussis* did not invade the tissues. However, recent studies have shown the bacteria to be present in alveolar macrophages.

5. Epidemiology

Pertussis occurs worldwide. Pertussis is a human disease. No animal or insect source or vector is known to exist. Adolescents and adults are an important reservoir for B. pertussis and are often the source of infection for infants. Transmission most commonly occurs by the respiratory route through contact with respiratory droplets, or by contact with airborne droplets of respiratory secretions. Transmission occurs less frequently by contact with freshly contaminated articles of an infected person. A silent carrier state is thought to exist, but it is infrequent, transient in duration, and probably of little importance in maintaining pertussis organisms in the community. Pertussis has no distinct seasonal pattern, but it may increase in the summer and fall. Pertussis is highly communicable, as evidenced by secondary attack rates of 80% among susceptible household contacts. Persons with pertussis are most infectious during the catarrhal period and the first 2 weeks after cough onset (i.e., approximately 21 days).

Pertussis is an endemic disease with epidemic peaks occurring every 2 to 5 (typically, 3 to 4) years. Widespread pertussis vaccination of children and the consequent reduction in the incidence of disease do not appear to have altered these intervals, suggesting that ongoing endemic circulation of the organism in the community continues. There is no consistent seasonal pattern.

Pertussis may occur at any age. Infants are susceptible to pertussis within the first few weeks or months of life, when mortality from whooping cough is highest. For many years, it was assumed that one attack of pertussis provided lifelong immunity. Before widespread vaccination, this belief was reflected by the age distribution of pertussis: approximately 20% of all whooping cough cases occurred in infants younger than 1 year, and nearly 60% occurred among children ages 1 to 4 years (Edwards et al., 2004).

Duchén et al. (1997) investigated about the response of immunoglobulin E and G to pertussis toxin in children immunized with adsorbed and non-adsorbed whole cell pertussis vaccines. The results showed that the adsorbed vaccine influenced the IgG response but not the IgE response to pertussis toxin.

Two classes of pertussis vaccine are currently available: whole-cell vaccines and acellular vaccines. The whole-cell vaccines are suspensions of killed *Bordetella pertussis* organisms at a concentration of more than 4 IU. The vaccine is adsorbed onto aluminium phosphate or aluminium phosphate sulphate (Clements et al., 2002).

IV. Japanese Encephalitis

Japanese encephalitis (JE) is the most important form of viral encephalitis in Asia, causing at least 50,000 cases of clinical disease and 10,000 deaths each year, mostly among children. In recent decades outbreaks of JE have occurred in several previously non-endemic areas, and the high fatality rate and frequent residual neuropsychiatric sequelae in survivors make JE a considerable public health problem in many Asian regions. Mosquitos transmit the JE virus from viraemic animals, mostly domestic pigs, to humans at seasonal intervals. There is no drug treatment for JE, and although improvements in agricultural practices have contributed to the reduction in disease incidence in some countries (Weekly Epidemiological Record, No. 44, 30 October 1998, 337-344).

During the first half of this century, JE was recognized principally in temperate areas of Asia in the form of perennial outbreaks in Japan, Korea, and China. In Japan, Korea, and Taiwan, the introduction of national immunization

programs after 1965 led to the near elimination of the disease; however, the absence of reported cases is disarming because enzootic transmission of the virus in its enzootic cycle continues in these locations, and periodic outbreaks, as in Korea in 1982, have occurred. Although sporadic viral encephalitis cases had been noted in northern Thailand, JE was not recognized as a major public health problem in Southeast Asia until 1969, when an epidemic of 685 cases was reported from the Chiang Mai Valley. Yearly outbreaks producing thousands of cases and hundreds of deaths followed in the northern region, and JE became recognized as a leading cause of childhood mortality and disability. The continued public health impact of JE in the region has led to efforts in Thailand and, more recently, in Vietnam to implement programs of childhood immunization and vaccine production (Halstead and Tsai, 2004; Rao, 2004).

1. Clinical description

The great majority of infections are not apparent, and only 1 in approximately 250 infections results in symptomatic illness in susceptible Asians. The principal clinical manifestation of illness is encephalitis. Milder clinical presentations, such as aseptic meningitis and simple febrile illness with headache, may sometimes occur but usually escape recognition. The incubation period is 5 to 15 days. Illness usually begins with abrupt onset of high fever, change in mental status, gastrointestinal symptoms, and headache, followed gradually by disturbances in speech or gait or other motor dysfunction. Irritability, vomiting, and diarrhea or an acute convulsion may be the earliest signs of illness in an infant or child. Seizures occur in more than 75% of pediatric patients and less frequently in adults.

A substantial proportion of patients become totally unresponsive and require ventilatory assistance. Generalized weakness and changes in muscle tone, especially hypertonia and hyperreflexia, are common, but focal motor deficits cranial nerve palsies (especially central facial palsy); and abnormal reflexes. Signs of extrapyramidal involvement, including tremor, mask like facies, rigidity, and choreoathetoid movements, are characteristic of JE, but these signs may be obscured initially by generalized weakness.

Five to 30% of cases are fatal, with some deaths occurring after a brief prodrome and fulminant course lasting a few days and others occurring after a more protracted course with persistent coma. Young children (< 10 years) are more likely than adults to die, and, if they survive, they are more likely to have residual neurologic deficits. Overall, approximately one third of surviving patients exhibit serious residual neurologic disability. In children, motor abnormalities frequently improve or eventually resolve, but behavioral changes and psychological deficits have been detected 2 to 5 years after recovery in up to 75% of pediatric cases (Halstead et al., 2004; Rao, 2004).

2. Virology

JE disease is caused by Japanese encephalitis virus (JEV), one of 73 viruses in the *Flavivirus* genus of the Flaviviridae family. Morphologically, flaviviruses are spherical, approximately 40-50 nm in diameter. The RNA genome is contained in a lipid membrane enclosing an isometric 30 nm diameter nucleocapsid core comprising a nucleocapsid or core protein (C; 12 kDa) and a single stranded messenger (positive) sense viral RNA. Membrane surface projections are composed of a glycosylated envelope protein (E; 53 kDa) and membrane protein (M; 8 kDa), a mature form of the premembrane (prM) protein in intracellular virion as shown in figure 2 (Heinz and Mandl, 1993; Halstead et al., 2004; Rao, 2004).

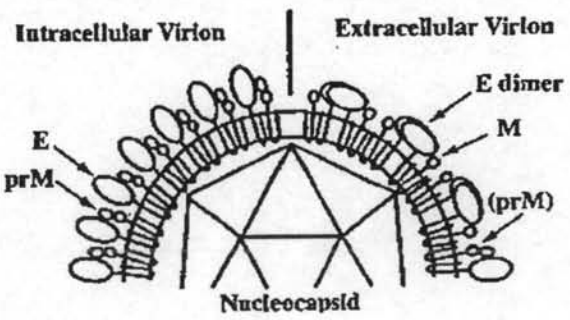

Figure 2 Morphology of flaviviruses cell membrane (Lindenbach and Rice, 2001).

3. Pathogenesis

After an infectious mosquito bite, viral replication occurs locally and in regional lymph nodes. Virions disseminate to secondary sites, where further replication contributes to a viremia. Invasion of the central nervous system (CNS) probably occurs from the blood by antipodal transport of virions through vascular endothelial cells. Infection in the CNS spreads by viral dissemination through the extracellular space or by direct intercellular spreads (Halstead et al., 2004).

4. Epidemiology

JE is transmitted in epidemics or in an endemic pattern, or both, in virtually every country of Asia. Transmission is seasonal, occurring approximately from May to September in temperate areas of China, Korea, Japan, and far eastern Russia. Farther south, the transmission season is somewhat longer, extending from March through October (Fig. 3). In tropical areas of Southeast Asia and India, seasonal transmission is particular to local patterns of monsoon rains and bird migration, with the possibility of two transmission intervals in a calendar year. The virus is transmitted throughout the year in some sites.

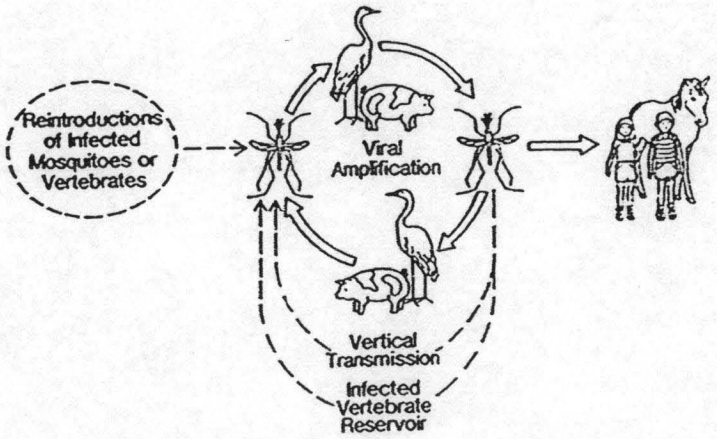

Figure 3 Transmission cycle of Japanese encephalitis (JE) virus. (Halstead et al., 2004)

JE is principally a disease of rural areas in which vector mosquitoes proliferate in close association with birds and pigs, which serve as vertebrate amplifying hosts. Humans and horses may become ill after infection, but such infections contribute minimally to the transmission cycle. Experimental observations and field studies indicate that the virus overwinters in infected adult mosquitoes. Long term persistence in tissues and blood of JEV infected vertebrate hosts, such as bats and reptiles, has been demonstrated. Although vector abundance and risk for human infection are associated with rainfall, with the introduction of wet rice cultivation, paddy flooding schedules have come to influence vector bionomics (Halstead et al., 2004).

In temperate regions, vector mosquitoes emerge in May, and, after several initial rounds of viral amplification, high rates of pig seroconversion are detected. This is followed almost immediately by the onset of human cases, typically in July and August. By virtue of high levels and lengthy periods of viremia after infection and their prevalence as domestic animals, pigs are the key hosts for viral amplification during pregnancy frequently results in abortions and stillbirths, with significant economic losses. In some locations, enzootic transmission of the virus is initiated among aquatic birds, and, in well characterized outbreaks in which pigs were absent, such birds have served as epidemic amplifying hosts. Other domesticated animals, such as cattle, dogs, sheep, cows, and chickens, and peridomestic rodents may become infected, but these fail to develop a sufficient viremia to support further viral amplification. JE mosquito vectors are zoophilic; consequently, cows and certain other animals can reduce risk to humans by diverting vector mosquitoes (zooprophylaxis). Immunization of pigs prevents abortion and stillbirths and also may reduce viral transmission by nullifying the role of pigs as viral amplifiers (Halstead et al., 2004; Rao, 2004).

The exposure and infection occur at an early age. In areas where transmission is hyperendemic, half of all cases occur in children younger than 4 years of age, and nearly all cases are found in children younger than 10 years. Usually cases in males exceed those in females, possibly reflecting greater outdoor exposure in boys.

Table 1 Japanese Encephalitis vaccines (Halstead et al., 2004)

Vaccine Type	Substrate	Viral Strains	Manufacturers
Inactivated	Mouse brain	Nakayama, Beijing-1(P1)	*India* : Central Research Institute (currently inactive) *Japan* : Biken (Research Foundation for Microbial Disease of Osaka University), Chiba, Denka Seiken Co.,Ltd., Chemo-Sero Therapeutic Research Institute, Kitasato Institute, Saikin Kagaku Institute, Takeda *Korea* : Green Cross *Taiwan* : National Institute of Preventive Medicine *Thailand* : Government Pharmaceutical Organization *Vietnam* : National Institute of Hygiene
Inactivated	Primary hamster kidney cells	P3	*People's Republic of China*: Beijing, Shanghai, and Changchun Institutes of Biological Products
Live, attenuated	Primary hamster kidney cells	SA14-14-2	*People's Republic of China*: Chengdu, Wuhan Institutes of Biological Products

Three JE control strategies have been considered based on transmission cycle of JE including vector control in mosquito, vertebrate reservoir immunization, and human immunization. The vector control in mosquito apparently decreases vector density but effect is just a moment. Although the vertebrate reservoir immunization, such as swine vaccination, is carried out in several regions, many problems have been recognized. Therefore, human immunization remains the most reliable control for JE infection at present.

Worldwide, three JE vaccines are in widespread production and use however, only inactivated JE vaccine produced in mouse brain is distributed commercially and is available internationally.

In most areas of Asia, vaccine produced from the Nakayama strain is given subcutaneously in two 0.5 ml doses 1 to 4 weeks apart (1.0 ml for people > 3 years of age) usually beginning at the age of 12 to 36 months, with a booster dose at 1 year and additional booster doses thereafter at 1 to 3 year intervals. In practice, immunization schedules are quite variable. Beijing 1 strain derived vaccine is formulated with a higher antigen concentration, and the recommended dose is 0.5 ml (0.25 ml for children under 3 years of age) (Halstead et al., 2004).

For traveler vaccination, the immunization consists of three doses on days 0, 7, and 30. An accelerated schedule might also be used. Doses are given on days 0, 7, and 14 can be used when departure is imminent. The vaccine's efficacy is 91% after two doses (Re and Gluckman, 2004).

The primary series has been administered to infants (with diphtheria and tetanus toxoids and pertussis [DTP] vaccine) as early as 2 months of age in clinical trials, but, because JE rarely occurs in infants younger than 1 year, there is no need to begin immunization at that age other than to save administration costs. In a study of infants 15 months of age, simultaneous administration of inactivated JE vaccine with measles, mumps, and rubella vaccine did not result in reduced immunogenicity of increased side effects. Under Thailand's Expanded Programme of Immunization, JE vaccine is given concurrently with the fourth dose of DTP and oral poliovirus vaccine at 18 months. A comparison of administration routes in adults showed that a 0.1 ml intradermal dose may be as immunogenic as the standard administration of 1.0 ml subcutaneously, at least when given as a booster (Halstead et al., 2004).

Rojanasuphot, Charoensook, Ungchusak, Srijaggrawalwong and Panthumachinda (1991) studied on the effectiveness of inactivated mouse brain JE vaccine produced in Thailand and in Japan in 5 to 9 year old children in Ratchaburi

Province; JE vaccines produced in Thailand are as effective as vaccines made in Japan.

In general, DTP vaccine has been given to children at the age of 2, 4 and 6 months and two booster doses at 18 months and 4 years, respectively, whereas JE vaccine has been given at 12-15 months and 2 years. Rojanasuphot, Na-Chiang Mai, Srijaggrawalwong, Panthumachinda and Nimmannitya (1992) investigated the possibility, safety and immunogenicity of implementing JE vaccination simultaneously with DTP and OPV vaccine in infants at Children's hospital, Bangkok.

V. Immunization program

Table 2 Immunization program (กุลกัญญา, 2545)

Age	Vaccine
New born	♦ BCG vaccine
	♦ Hepatitis B vaccine (1st dose)
1 month	♦ Hepatitis B vaccine (2nd dose)
2 months	♦ DTP, OPV vaccine (1st dose)
4 months	♦ DTP, OPV vaccine (2nd dose)
6 months	♦ DTP, OPV vaccine (3rd dose)
	♦ Hepatitis B vaccine (3rd dose)
9 months	♦ MMR vaccine (1st dose)
12-15 months	♦ JE vaccine (1st and 2nd dose in 1-2 weeks interval)
18 months	♦ DTP, OPV (1st booster)
2 years	♦ JE vaccine (3rd booster)
4 years	♦ DTP, OPV vaccine (2nd booster)
5-6 years	♦ MMR vaccine (2nd dose)

It could be concluded that JE vaccination simultaneously with routine vaccines was safe, effective and practical. Two-and three- month intervals of a primary two dose JE vaccination were not different from the seven-day interval in inducing immunogenicity. Even the antibody one year after 2 doses of JE vaccination remained sufficiently high to confer protection against JE infection: the third dose vaccination also had a marked effect on antibody response. Srivastava et al. (2001) formulated a second generation, purified, inactivated vaccine (PIV) against JE virus with aluminium hydroxide and administered to mice by subcutaneous inoculation and to compare with the existing licensed mouse brain-derived vaccine, JE-Vax. The JE-PIV was more immunogenic than and as effective as preventing encephalitis in mice.

VI. Adjuvants

Novel approaches of vaccine delivery system have been developed. Recombinant DNA technology, produced of a variety of subunit vaccine, gives significant advantages over more traditional vaccines. However, a number of these vaccines have been encountered to be nonimmunogenic or weakly immunogenic because of highly purified antigens and small molecular structures (Zhao, 1996). Then, the use of potent adjuvants are required to induce a vigorous immune response.

Adjuvants are described as substances used in combination with a specific antigen that generated more immunity than antigen alone (O' Hagan, 1997). Some desirable general properties of adjuvants are listed in Table 3.

1. Mechanism of action of adjuvants

Adjuvants can also perform in one or more five mechanisms as concluded in Table 4 (Gupta and Siber, 1995; Cox and Coulter, 1997; Kenny and Edelman, 2004).

1.1 Immunomodulation

This mechanism refers to ability to modify cytokine network. Immunomodulation may result in a general upregulation of entire immune system,

that most commonly results in upregulation of certain cytokines and concomitantly down regulation of others. Two major subsets of T cell, Th1 and Th2 have involved in this mechanism. Th1 responses typically induce complement fixing antibody and strong delayed-type hypersensitivity (DTH) reactions. Th2 responses result in high circulation and secretory antibody levels. However, Th1 and Th2 responses are mutually impediment.

Immunomodulatory adjuvants not only lead to increase immune response but also determine IgG isotype which other immunoglobulins and how much T cells directed, so cell mediated immunity is generated.

Table 3 Some desirable general properties of adjuvants

Safety
It must not be carcinogenic, teratogenic, or abortogenic.
The formation of granulomas, local necrosis, hypersensitivity, fever, or autoimmune effects should be avoided.
Nonspecific effects on cell activation, caused by perturbation of cell membranes by surfactants or oils, should be avoided.
It should be biodegradable and preferably have a short half-life.
Specificity
Because most activation signals may be transduced by membrane phospholipase activation, the activity should be targeted to specific cells of the immune system. Such cells may already posses specific receptors, especially if the adjuvant is derived from an infectious agent.
Having a known chemical structure is desirable.
Feasibility and formulation
The preparation should be stable, inexpensive, simple to (reproducibly) manufacture, and have a long storage life.
Presentation of the antigen with adjuvant as particles with multimeric arrays of antigen is advantageous.

1.2 Presentation

Presentation means ability to preserve conformational integrity of an antigen and to present to suitable immune effector cells. This ability happen when an adjuvant is able to interact with an antigen in such a way that conformational epitopes are more effectively maintained. Hence, the major advantages of antigen presentation are an improved amount of conformationally relevant antibody such as neutralizing antibody, an increased affinity of antibody and duration of immune response.

1.3 Induction of cytotoxicT-lymphocyte (CTL) responses

The CTL induction responses usually require that antigen be processed within cytosol cell (endogenous pathway) where peptides become incorporate within close-end groove of major histocompatibility class 1 (MHC-1) molecule and are then expressed on cell surface.

An adjuvant to be used for CTL induction must facilitate incorporation or persistence of appropriate peptide into MHC-1. The adjuvant interact with cell membrane so that antigen associated with adjuvant is deposited within cytosol cell. Although most cells express MHC-1, the most effective target cell for CTL induction is an antigen presenting cells (APCs) and most probably a typically dendritic cells (DC).

1.4 Targeting

This defines the ability of an adjuvant to deliver an immunogen to immune effector cells, generally via APCs. This form of adjuvant activity may not modify the type of immune response but rather will affect the amount of immunogen required to achieve a given effect that is the efficiency of generation of the immune response. There are several ways in which an adjuvant can achieve this effect. The most common is to interact antigen to form multimolecular aggregates, which encourage uptake by macrophages and DC, and deliver antigen to APCs. Adjuvants with this property are called particulate adjuvants.

Table 4 Mode of adjuvant action (Cox et al., 1997)

Action	Adjuvant type	Benefit
Immunomodulation	Generally small molecules or proteins which modify the cytokine network	Upregulation of immune response. Selection of Th1 or Th2
Presentation	Generally amphipathic molecules or complexes which interact with immunogen in its native conformation	Increased neutralizing antibody response. Greater duration response
CTL induction	- Particles which can bind or enclose immunogen and which can fuse with or disrupt cell membranes	Cytosolic processing of protein yielding correct class 1 restricted peptide
	- w/o emulsions for direct attachment of peptide to cell surface MHC-1	Simple process if promiscuous peptide know
Targeting	- Particulate adjuvants which bind immunogen. Adjuvants which saturate Kupffer cells	Efficient use of adjuvant and immunogen
	- Carbohydrate adjuvants which target lectin receptorson macrophages and DCs	As above. May also determine type of response if targeting selective
Depot generation	- w/o emulsion for short term	Efficiency
	- Microspheres or nanospheres for long term	Potential for single-dose vaccine

1.5 Depot generation

This can be achieved as a short term or long term depot. Short term depots are typified by aluminium salts and w/o emulsions, where antigen is trapped at the injection site and therefore cannot be lost by liver clearance. Excision of the injection site 8-10 days after dosing has little if any effect on magnitude or duration of response suggesting antigen has either been removed or walled-off by that stage.

Long term depots are best achieved using synthetic polymers such as polylactide coglycolide (PLG) to produce microspheres which degrade to yield a pulsed delivery. These microspheres are preferably of a size >10 μm so that they must remain at the injection site until biodegradation permits removal of their contents (immunogen and preferably adjuvant) by APC. Release times from 1 to 6 months can be achieved with reasonable precision.

2. Classification of adjuvants

The successful development of adjuvants requires consideration of a number of issues. The characteristics of an ideal vaccine adjuvant are shown in Table 5. The safety is the most substantial issue to be considered. Other considerable issues are including stability, ease to produce, a wide range applicability and cost. Moreover, an ideal adjuvant of vaccine should be capable of being administered with a wide range of antigens by a variety of different routes, such as oral, parenteral or intranasal.

There are a great number of different criteria which can be used to classify vaccine adjuvants. Gupta et al. (1995) and Cox et al. (1997) have been allocated adjuvants into two broad groups, particulate and nonparticulate adjuvants, as briefly described below.

2.1 Particulate adjuvants

The substances which exist as microscopic particles and owe at least some of their adjuvant activity to this property. The particulate adjuvants as denoted in Table 6 are advantages when immunogen is able to be incorporated into or at least associated with the particle.

2.2 Non-particulate adjuvants

Non-particulate adjuvants are adjuvants where activity does not depend on any particulate or multimeric nature. In some instances, they can afford good immune responses, generally, through immunomodulation action but some improve

targeting. The characteristics of principal and other non-particulate adjuvants are summarized in Table 7 and 8.

Table 5 The characteristics of an ideal vaccine adjuvant

Biodegradable and biocompatible
Should not be toxic, carcinogenic, teratogenic or abortogenic
Non-antigenic and not immunologically cross-reactive with tissue antigens
Induce a minimum of injection site reactogenicity
Simple well defined chemical structure
Induce a minimum of non-specific effects on immune system
Acceptable for administration to man
Safe to administer to young and immunocompromised individuals
Effective for peptide, protein, polysaccharide and DNA
Effective after a single dose
Induce both humoral and cell-mediated immunity
Capable of being administered orally
Induce systemic and mucosal immunity
Promote antigen uptake by lymphoid tissues
Stable formulation which is inexpensive to manufacture
Can be manufactured reproducibly on a large scale
Good shelf-life, preferably without refrigeration
Easy to mix with antigen or combination of antigens

Table 6 Characteristics of particulate adjuvants (Cox et al., 1997)

Adjuvant	Immunomodulation	Targeting	Presentation	CTL	Depot
Aluminium salts	Strong Th2, IgE	+	-	-	+ST[a]
w/o emulsions	Weak Th1 and Th2	-	-	- or +++[b]	+++ST
o/w emulsions	Weak Th1 and Th2	+	+++	-	-
ISCOM™	Strong Th1 and Th2	+++	++++	++++	-
Liposomes	-	++	+++	++	-
Microparticles					
< 10 µm	-	++++	-	-	-
> 10 µm	-	-	-	-	+++LT[c]
Calcium salts	-	+	-	-	+ST
Proteosomes / virosomes	-	++	+++	-	-
Stearyl tyrosine	Mod Th1 and Th2	-	-	-	+ST
γ-Inulin	Mod Th1	-	-	-	-
Algammulin	Mod Th1 and Th2	+	-	-	+ST

[a] ST, short term(≤ 2 weeks); [b] Good CTL response for externally applied peptide only;
[c] LT, long term (weeks to months)

Table 7 Characteristics of principal non-particulate adjuvants (Cox et al., 1997)

Adjuvant	Immunomodulation	Targeting	Presenting	CTL	comments
MDP-hydrophilic	Strong Th2	-	-	-	use in w/o emulsions
MDP-lipophilic	Strong Th1	-	-	-	use in o/w emulsions
Non-ionic block copolymers	?	- or +++[a]	+++	-	use in w/o or o/w emulsions
saponins	Strong Th1, Th2	-	-	+	from ISCOMs, use with liposomes, MPL
Lipid A (MPL)	Strong Th1	-	-	-	use with o/w emulsions, liposomes, saponins
Cytokines	Various	-	-	-	use preferably with some particulate adjuvant
Carbohydrate polymers	Mod Th1, IL-1 induction	+++	-	-	preferably conjugate?
Derivatized polysaccharides	?	+++	-	-	

[a]For self-aggregating copolymers, e.g. CRL 1005

Table 8 Further non-particulate adjuvants (Cox et al., 1997)

Adjuvant	Action	Comments
Avridine DNA	Th1 induction, Presentation (in liposome or o/w emulsions)	Unacceptable toxicity
CWS (cell wall skeleton)	Th1 induction ?	Use with MPL in o/w emulsions
DHEA (dehydroepi-androsterone)	Th1 induction ?	Administration difficult
Vitamin D3	Th2, secretory IgA induction ?	Administration difficult
TDM (Trehalose dimycolate)	Th1 induction	Administration difficult Toxicity unacceptable
P_3CSS	Targeting, potent CTL induction	Potentially toxic
Poly I:CPoly ICLC	Both Th1 (γIFN) and Th2 (IL-4) induction	Poly I:C toxic
Poly A:U	Th2 induction (IL-6)	

3. Safety

The absolute safety of adjuvanted vaccines, or any vaccine, cannot be guaranteed, so the risks must be minimized. Undesirable reactions can be grouped as either local or systemic. The most frequent local adverse effects are tenderness and swelling, with the most severe ones involving the formation of painful abscesses and nodules at the inoculum site. The mechanisms for such severe local reactions include the following: 1) contamination of the vaccine at the time of formulation with reactogenic chemicals and microbial products; 2) instability of the vaccine on storage with breakdown into reactogenic side products; 3) formation of inflammatory immune complexes at the inoculation site by combination of the adjuvanted vaccine with preexisting antibodies resulting in an Arthus-type reaction; and 4) poor

biodegradability of the adjuvanted vaccine resulting in prolonged persistence in the tissues and reactive granuloma formation. Such local reaction are of special concern for depot-type adjuvants, such ad aluminum salts, oil emulsions, liposomes, biodegradable polymer microspheres, and living vectors such as BCG. To date, vaccine adjuvants have caused few severe acute systemic adverse effects (Kenny et al.; 2004).

4. Aluminium adjuvants

The adjuvant action of aluminium compounds was first noted in 1926 when an alum-precipitated diphtheria vaccine was found to have greater antigenic properties than the standard diphtheria vaccine. Aluminium compounds, including aluminium phosphate ($AlPO_4$), aluminium hydroxide ($Al(OH)_3$) and alum precipitated vaccines, historically referred to as protein aluminate, are currently the most commonly used adjuvants with human and veterinary vaccines. These adjuvants are often referred to as "alum" in the literature, which is misleading, because (1) alum, chemically potassium aluminium sulfate ($KAl(SO_4)_2.12H_2O$); (2) aluminium hydroxide and aluminium phosphate have different physical characteristics and differ in their adjuvant properties. Alum was originally used to partially purify protein antigens, mainly tetanus and diphtheria toxoids by precipitating them in the presence of anions including phosphate, sulphate, and bicarbonate ions resulting in a mixture of compounds, mainly aluminium phosphate and aluminium hydroxide (Gupta et al., 1995; Gupta, 1998; Baylor et al., 2002).

4.1 Physico-chemical characteristics

Aluminium in the form of aluminium hydroxide, aluminium phosphate or alum continues to be commonly used as an adjuvant in vaccines. Aluminium hydroxide has been identified as crystalline aluminium oxyhydroxide with a structure of the mineral boehmite. It has high surface area with an isoelectric point (pI) of 11 that is positively charged at physiological pH. In contrast, aluminium phosphate has been classified as amorphous aluminium hydroxyphosphate which is negatively charged at physiological pH (pI = 4-7). In alum-precipitated vaccines, alum is an

aluminium hydroxide that contains some sulfate anions as well as anions that are used in the buffer, often phosphate. The pI depends on the precipitation process and is usually in the range of 0.3-0.6. The amorphous nature of aluminium phosphate contributes to high surface area and high protein adsorption capacity, mainly for positively charged proteins. That is the reason for poor adsorption of negatively charged diphtheria toxoid onto aluminium phosphate at neutral pH (Gupta, 1998; Baylor et al., 2002; Matheis et al., 2002; Lindblad, 2004).

4.2 Adjuvant properties

The adjuvanticity of aluminium adjuvants for human vaccines, particularly tetanus and diphtheria toxoids, was clearly established during the 1930's. The major advantage of using aluminium adjuvants was the more rapid development of high titered and long-lasting antibody responses after primary immunization. There are numerous reports in humans and animals showing the superiority of aluminium adsorbed tetanus and diphtheria toxoids over soluble toxoids, particularly after the first dose. Aluminium adjuvants are universally used with diphtheria, tetanus and pertussis (DTP) vaccines, although, the adjuvant effect on whole cell pertussis component is not clear. Although serum agglutinins to *Bordetella pertussis* produced after immunization with aluminium adjuvanted pertussis vaccine were higher than those obtained after inoculation with unadsorbed pertussis vaccine, there was no difference between unadsorbed and adjuvanted pertussis vaccine with regard to protective against disease. In a few studies, the potency of adjuvanted vaccine was higher than the non-adjuvanted pertussis vaccine but in other studies, the adjuvant did not have any effect. Aluminium compounds are routinely used with the new acellular pertussis vaccines and which have been used with inactivated poliovaccine, human diploid cell strain rabies vaccine, hepatitis B vaccine, hepatitis A vaccine, cholera vaccine and Hib conjugate vaccine.

Aluminium adjuvants have also been widely used with a number of veterinary vaccines, including vaccines against avian infectious bronchitis, canine hepatitis, foot and mouth disease, Newcastle disease, *Bacteroides nodosus*, *Bordetella bronchiseptica*, *Pasteurella multocida*, *Leptospira interrogans*, *Cooperia punctata*,

Nematospiroides dubius, Trichinella spiralis. Thus, aluminium adjuvants have wide applications with both human and veterinary vaccines (Gupta, 1998).

4.3 Adsorption on adjuvanticity

The immunogenicity of antigens adsorbed onto aluminium adjuvants appears to depend on the degree of antigen adsorption and the dose of adjuvant. The formulation which did not show any adsorption of diphtheria toxoid onto aluminium phosphate, due to presence of a ten-fold excess of phosphate, did not elicit an antibody response after the first injection and only a poor response after the second dose. The adsorption is considered to be a very important parameter for the function of these adjuvants (Gupta, 1998). Gupta et al. (1995) reported that the optimal pH for adsorption of tetanus and diphtheria toxoids onto aluminum phosphate was 6.0-6.3. For aluminium hydroxide in neutral aqueous solution such conditions are met with bovine serum albumin (BSA, pI 4.8) that, therefore, is often used as a model protein for determination of the adsorption properties of aluminium hydroxide. For the same reason lysozyme (pI 11) is used as a model protein for aluminium phosphate with a low pI (Matheis et al., 2002).

4.4 Dose of aluminium adjuvants

Aluminium compounds are the only adjuvants used in the manufacture of currently licensed vaccines in the United States Chapter 21 of the US Code of Federal Regulations [610.15(a)] governs the amount of aluminium permitted in the recommended single human dose of a product. The amount of aluminium is limited to no > 0.85 mg/dose if the level is assayed. The regulations were amended in 1981 to increase the permissible level of aluminium to 1.25 mg in biological products to make the regulations consistent with the World Health Organization standards per single human dose of a product. If aluminium compounds other than alum are used, the total amount of alum should not be more than the equivalent permitted as potassium alum (Gupta, 1998; Baylor et al., 2002).

4.5 Elimination

Aluminium is not biodegradable which have been found at site of subcutaneous injection in mice and guinea pigs for up to one year (Gupta, 1998). Keith et al. (2002) found 66-70% of injected aluminium was excreted in 24 hours. In a human study, Keith et al., 2002 reported that the volunteer was injected with 0.7 μg of radioactive ^{26}Al as citrate and followed blood levels and body elimination. They found that over 50% of the aluminium distributed from blood to other body tissues in 15 minutes. Long-term observation using excreta and whole body monitoring founds excretions of >50% in 24 hours, 85% at 13 days, and 96% by 1178 days. Aluminium containing adjuvants which are administered intramuscularly are dissolved in intestinal fluid, absorbed into the blood, distributed to tissues and eliminated in urine (Hem, 2002).

Verdier et al. (2005) investigated the clearance of aluminium at the vaccine injection site and the features of induced histopathological lesions. They concluded that aluminium adjuvanted vaccines administered by the intramuscular route trigger histopathological changes restricted to the area around the injection site which persist for several months but are not associated with abnormal clinical signs.

4.6 Limitations

Aluminium adjuvants cannot be frozen or easily lyophilized as both of these processes cause the collapse of gel resulting in gross aggregation and precipitation. Although tetanus toxoid with collapsed gel precipitates was found to be immunogenic, such a vaccine is not clinically acceptable. Successful lyophilization of aluminium adjuvants was reported but lyophilized vaccines containing adjuvants are not available commercially. The immunogenicity of antigens adsorbed onto aluminium adjuvants depends on several factors; however, the most important is the degree of adsorption of antigen on the adjuvant and the dose of adjuvant. As far as dose, a small amount of adjuvant may be required for complete adsorption. Even though small doses may completely adsorb the antigens, they may not show an optimal adjuvant effect. Excessive amounts of aluminium compounds may suppress

immunity by covering the antigen completely with mineral compounds or the aluminium compounds may be cytotoxic to macrophages. Aluminium hydroxide has been demonstrated to have a more potent adjuvant effect than aluminium phosphate which may be due to its higher adsorption capacity and better adsorption of certain antigens at neutral pH. (Gupta, 1998; Lindblad, 2004)

4.7 Safety

Aluminium-containing vaccines have been associated with severe local reaction such as erythema, subcutaneous nodules, contact hypersensitivity and granulomatous inflammation. Some studies with aluminium-adsorbed DTP vaccine have reported fewer reactions than unadsorbed vaccine. Aluminium hydroxide has been reported to attract eosinophils to the injection site, and may increase the levels of antigen-specific and total IgE antibodies that may promote IgE-mediated allergic reactions. On the other hand, aluminium adjuvants have been used for years for hyposensitization of allergic patients without adverse results. There have also been reports, especially in patients with impaired renal function, of systemic accumulation of aluminium, which has been associated with nervous disorders and bone disease. Nonetheless, aluminium intake from vaccines is far less than that received from the diet or medications such as antacids (Baylor et al., 2002).

4.8 Comparative adjuvanticity of aluminium compounds

Aluminium hydroxide has been found to be a more potent adjuvant than aluminium phosphate. This may be due to its overall higher adsorption capacity and better adsorption properties of certain antigens at neutral pH. Aluminium hydroxide adjuvanted antigens induced antibody responses that are comparable to Freund's Complete Adjuvant (FCA). Aluminium hydroxide is a good adjuvant for weak immunogens. Aluminium compounds are also very potent adjuvants for tetanus and diphtheria toxoids in guinea pigs and mice (Gupta, 1998; Baylor et al., 2002)

Shi, HogenEsch, Regnier and Hem (2001) investigated adsorption isotherms of endotoxin and aluminium containing adjuvants at pH 7.4 and 25 °C

revealed that aluminum hydroxide adjuvant has a greater adsorption capacity (283 µg/mg Al) than aluminium phosphate adjuvant (3.0 µg/mg Al) and the difference in endotoxin adsorption was related to two adsorption mechanisms: electrostatic attraction and covalent bonding.

4.9 Aluminium content in currently licensed vaccines

As previously stated, the US FDA (21 CFR 610.15(a)) allows no > 0.85 mg/dose of aluminium in vaccines, the currently licensed vaccines use alum, aluminium hydroxide, aluminium phosphate, or a combination of aluminium hydroxide and aluminium phosphate. The total amount of aluminium received from vaccines will vary depending on which brand of vaccine is given. A 1-year-old who receives a complete series of recommended vaccines may receive a minimum of 1.6 mg of aluminium or a maximum of 4.1 mg of aluminium from these vaccines. A 5 — year-old would be exposed to a similar amount of aluminium, 1.9 - 4.9 mg, if the recommended vaccines for this age were received. The amount of aluminium an adult would receive from vaccines varies greatly depending on the number of vaccines given (Baylor et al., 2002).

Analysis of aluminium content of aluminium hydroxide gel has been reported employing various methods such as chelatometric titration (Nail et al., 1976; Masood, White and Hem, 1994; Burrell et al., 2000; Chang et al., 2001; BP 1998; USP 25) inductively coupled plasma (ICP) spectroscopy (May et al., 1984; Burrell et al., 2001) and atomic adsorption spectrometry (AAS) (May et al., 1984; Rinella et al., 1998; Lindblad, 2004).

Table 9 Aluminium content of licensed vaccines (Baylor et al., 2002)

Vaccine	Trade name	Source	Al per dose (μg)	Chemical form of Al	No. of dose in series(mg)	Total Al for series
Childhood vaccines						
DTaP	Infanrix	SKB	≤625	Hydroxide	5	3.1
	Certiva	NAVA	500	Hydroxide	5	2.5
	Acelimune	Lederle	230	Hydroxide/ Phosphate	5	1.2
	Tripedia	Avent. Past Inc.	≤170	Alum	5	0.85
DTP	-	Bioport	≤600	Phosphate	5	3.0
	-	Avent. Past Inc.	≤170	Alum	5	0.85
Hib conjugate	Liq. Pedvax Hib	Merck	225	Hydroxide	3	0.68
Pneumo conjugate	Prevenar	Lederle	125	Phosphate	3	0.38
DTP-Hib	Tetramune	Lederle	≤850	Hydroxide	4	3.4
Hep B-Hib	Comvax	Merck	225	Hydroxide	3	0.68
Hep B	Recombivax B	Merck	225	Hydroxide	3	0.68
	Engerix B	SKB	250	Hydroxide	3	0.75
DT, adsorbed	-	Avent. Past Inc.	≤170	Alum	5	0.85
	-	MPHBL	450	Phosphate	5	2.3
	-	Bioport	≤600	Phosphate	5	3.0
	-	Lederle	≤800	Phosphate	5	4.0
	-	Wyeth	≤850	Phosphate	5	4.3
Adult vaccines (some may also be indicated for younger age groups)						
T, adsorbed	-	Lederle	≤850	Phosphate	6	5.1
	-	MPHBL	450	Phosphate	6	2.7
	-	Wyeth	≤850	Phosphate	6	5.1
	-	SSVI	≤850	Phosphate	6	5.1
	-	Avent. Past Inc.	≤250	Alum	6	1.5
Td, adsorbed	-	Lederle	<800	Phosphate	6	4.8
Td, adsorbed	-	Wyeth	≤850	Phosphate	6	5.1
	-	PHBL	450	Phosphate	6	2.7
	-	Avent. Past Inc.	≤280	Alum	6	1.7
Hep A	Havix	SKB	250	Hydroxide	2	0.5
	VAQTA	Merck	450	Hydroxide	2	0.9
	VAQTA	Merck	225	Hydroxide	2	0.45
Lyme	Lymerix	SKB	≤500	Hydroxide	3	1.5
Anthrax	Lymerix	BioPort	≤830	Hydroxide	6	5.0
Rabies	RabAvent	BioPort	442	Phosphate	5	2.2

VII. Combination vaccine

A combination vaccine consists of two or more immunogens which are physically combined and administered at the same time in the same anatomic site. The use of combination vaccines is one mechanism by which the number of injections can be reduced without reducing the number of diseases against which a child is protected. In addition to reducing the number of injections (and therefore the amount of trauma and pain experienced by the recipient), use of combination vaccines might improve the timeliness of vaccination coverage, reduce costs associated with stockpiling and administering separate vaccines, reduce costs associated with extra health care visits that result from delayed vaccinations, and facilitate the integration of new vaccines into the childhood immunization schedule (Postema, Myers and Breiman, 2001). These problems have stimulated continuing efforts to develop new combination vaccines.

The combining of multiple related or unrelated antigens into a single vaccine is not a new concept. Most such pediatric combination vaccines begin with a DTwP or DTaP vaccine and add such antigens as IPV, conjugate Haemophilus influenzae type b (Hib), and hepatitis B (HB). As development efforts for the DT(a)P based combinations have matured, some manufacturers have turned their efforts toward developing so called second shot combinations that incorporate conjugate pneumococcal (PnC) and conjugate meningococcal (MnC) antigens. A third developmental stream has been directed toward combination vaccines targeted principally travelers, typically based on HB or hepatitis A (HA) components (Decker, Edwards and Bogaerts, 2004).

Mallet et al. (2004) reviewed of the immunogenicity and safety of hexavalent vaccine (Hexavac®) which was comparable with following concomitant administration of Pentavac® and monovalent hepatitis B vaccine. Hexavac® shown to be highly immunogenic and could be used by vaccination schedule.

The premium pricing of the combination or reduced provider reimbursement (as a result of administration of fewer injections) may inhibit use of the combination. Less obvious, but equally important, are the economic benefits that flow from use of a combination: savings resulting from simplified vaccine purchase, storage, and handling ; reduced costs for labor and supplies ; elimination of the need for scheduling several vaccination visits to avoid multiple injections ; and, of course increased patient satisfaction and greater compliance with vaccination recommendations (Ada, 1994).

1. Source of interference between different vaccine preparations

1.1 Antigen competition

Ada (1994) reported that antigen competition may arise if the peptides from different protein antigens compete with binding to a particular major histocompatibility (MHC) molecule together with the differential recognition of those complexes by the T cell receptor (TCR). One peptide, A, may preferentially bind to a given MHC molecule compared to another peptide, B. Both the MHC/A and MHC/B complexes may be seen equally well by TCRs, but as there are now very few MHC/B complexes to be recognised, there will be a poor response to the protein supplying peptide B. For example, immunizing mice with myelin basic protein in the presence of ovalbumin results in a much decreased response to the myelin basic protein compared to the normal level obtained in the absence of ovalbumin.

It is usually not possible to predict such interference in advance. Theoretically, it becomes more likely as the number of proteins in a mixture increases. It may in part be overcome by administration of different components at different sites-not an ideal practical solution.

1.2 The effect of different adjuvants

They may be cases where for example a mucosal immune response (Th2) to one vaccine component of a mixture would be beneficial whereas a systemic

response favouring a Th1 response is most advantageous for another component and a cytotoxic T-lymphocyte (CTL) response to a third component of the mixture (Ada, 1994). Careful thought would need to be paid to the choice of adjuvant, but it is unlikely optimum responses to each would be obtained.

1.3 Interference with the replication of different infectious agents

It is well known that in the case of the three subtypes of attenuated polioviruses, adjustments to the amount of each subtype in the final vaccine need to be made to suit regional circumstances. A similar effect has recently been noticed with mixtures of cold adapted, live influenza virus vaccines. The factors that are potentially important in these or other cases are the varying susceptibilities of different cells to infection, the rates of replication of the different infectious agents and a variety of host factors, such as malnutrition, the extent of existing infections by other agents, etc (Ada, 1994).

VIII. Adsorption process for combined vaccines

The term adsorption describes attractive interactions at surfaces without formation of covalent chemical bonds. If covalent bonds are formed, the process is called chemisorption. In the case of vaccine production, adsorption means the attraction between antigens dissolved in the medium and colloidal dispersed adsorbent particles (aluminium hydroxide or aluminium phosphate). Two primary mechanisms of antigen adsorption to aluminium containing adjuvants are the electrostatic attractive force and ligand exchange, and the adsorption mechanism of hepatitis B surface antigen (HBsAg) is predominantly due to ligand exchange (Morefield, Jiang, Romero-Mendez, Geahlen, HogenEsch and Hem, 2005). Electrostatic as well as hydrophobic interactions play an important role in this process (Matheis, Zott and Schwanig, 2002).

Seeber, White and Hem (1991) studied the adsorption of two model proteins, albumin and lysozyme, by boehmite or amorphous aluminium hydroxyphosphate adjuvants. Electrostatic attraction has a major role in adsorption. At physiological pH,

boehmite, which has a point of zero charge above 7.35, extensively adsorbed albumin but was not effective in adsorbing lysozyme. Conversely, amorphous aluminium hydroxyphosphate was effective in adsorbing lysozyme but adsorbed relatively little albumin. The results suggested that the selection of either boehmite or amorphous aluminium hydroxyphosphate as an adjuvant should be based in part on the isoelectric point of the antigen.

Al-Shakhshir, Regnier, White and Hem (1994) examined the effect of adsorbing two model proteins, BSA and lysozyme, on the point of zero charge of aluminium-containing vaccine adjuvatns. At physiological pH, the adsorption of the negatively charged albumin (pI = 5.0) by aluminium hydroxide adjuvant resulted in a decrease in the point of zero charge. In contrast, the adsorption of positively charged lysozyme (pI = 9.6) by the negatively charged aluminium phosphate adjuvant resulted in an increase in the point of zero charge. The surface charge characteristics of the aluminium containing adjuvants dominated at low protein coverage. In contrast, the surface charge characteristics of the adsorbed protein dominated at high protein coverage.

The study of Jiang et al. (2004) showed that calcium phosphate adjuvant which is a commercially available vaccine adjuvant identified commercial calcium phosphate adjuvant as non-stoichiometric hydroxyapatite. The surface charge was pH-dependent. It exhibited a negative surface charge at physiological pH and electrostatically adsorbed positively charged antigens. The presence of hydroxyls allows calcium phosphate adjuvant to adsorbed phosphorylated antigens by ligand exchange with surface hydroxyls.

Iyer, HogenEsch and Hem (2003) investigated the effect of the degree of phosphate substitution in aluminium hydroxide adjuvant on the adsorption of phosphorylated proteins. The phosphorylated proteins (alpha casein, dephosphorylated alpha casein and ovalbumin) were adsorbed by ligand exchange of phosphate for hydroxyl even when an electrostatic repulsive force was present.

Iyer, Robinett, HogenEsch and Hem (2004) studied the mechanism of adsorption of hepatitis B surface antigen (HBsAg) by aluminium hydroxide adjuvant. The adsorption of HBsAg by aluminium hydroxide adjuvant exhibited a high affinity adsorption isotherm. The relatively high value of the adsorptive coefficient indicated that adsorption was due to a strong attractive force. Ligand exchange between a phosphate of the antigen and a surface hydroxyl of the adjuvant provided the strongest adsorption mechanism. The adsorption capacity of HBsAg was not affected by increased ionic strength indicating that electrostatic attraction was not the predominant adsorption force.

The adsorptive characteristics of recombinant protective antigen (rPA) and two aluminium containing adjuvants were examined in a physiological buffer with and without EDTA. It was predicted and demonstrated that rPA bound in a more efficient manner to aluminium hydroxide adjuvant than to aluminium phosphate adjuvant in the physiological buffer and the binding of the rPA to the aluminium hydroxide was decreased by increased amounts of phosphate in the buffer. These data suggested that the interaction between rPA and aluminium hydroxide was predominantly electrostatic in character (Jendrek, Little, Hem. Mitra, and Giardina, 2003).

Interactions between surfaces are strongly dependent on the surface charges of the interacting partners. Overall surface charges are quantified by measurement of the pI. This parameter is defined by the pH-value at which the overall surface charges are zero. For proteins the pI is determined by the motion of the molecule through a pH-gradient in an isoelectric focusing experiment (Matheis et al., 2002; Vogel and Hem, 2004).

Interactions between proteins and adsorbents are optimal, when their pIs differ and the pH of the medium is in between. In this case, protein and adsorbent have opposite surface charges. The diphtheria toxoids and tetanus toxoids show pI below pH 7. Therefore, the adsorbent aluminium hydroxide should be the best choice for these antigens (proteins). In some licensed vaccine preparations they are adsorbed on aluminium phosphate or on both adsorbents. Adsorption experiments with purified

diphtheria toxoids or tetanus toxoids are used in vaccines, showed complete adsorption of these toxoids on aluminium hydroxide. In the case of aluminium phosphate only partial adsorption is observed, even with toxoids concentration as low as in administered vaccine (Gupta, 1998; Matheis et al., 2002; Vogel, 2004).

Gupta et al. (1998) reported that the pH and ionic strength affect adsorption by altering charge on the gel and the antigen, whereas the temperature may affect the rate of interaction between the gel and the antigen. Size of gel particles affects the surface area of gel available for adsorption: small particles have more surface area than large particles. In addition, adsorption of diphtheria toxoid resulted in higher adsorption than commercial aluminium phosphate preparation, probably due to trapping of some antigen in the gel.

The adsorption/elution behavior could be explained by the effect of pH on: (1) the ionization state of the protein, (2) the solubility of the adjuvant, and (3) the electrostatic interaction between the protein and adjuvant (Rinella, White and Hem, 1998).

1. Factor affecting adsorption (Matheis et ai., 2002)

1.1 pH value

The pH value of the medium determines the real surface charge of antigen and adsorbent and, therefore, is important for the adsorption process. Practical experience showed that best results for adsorption could be obtained, when the pH is set near the pI of antigen. In this case, the interactions of antigen molecules are minimized and interaction with the adsorbent is effective. For long lasting adsorption the final pH should be between the pIs of antigen and adsorbent, respectively. If an antigen is already adsorbed and the adsorbed amount is not close to the maximum possible, small pH changes will not cause desorption. Therefore, in vaccine production the adsorption process for acidic antigens like diphtheria toxoids and tetanus toxoids be carried out at a pH below 7. For the final formulation the pH can be adjusted to a physiologically advantageous value around pH 7.

1.2 Ions

There are several ions in vaccine formulation that influence the adsorption capacity if they are present before, and may lead to desorption if added after adsorption. For example, highly charged ions like phosphate, sulphate, carbonate or citrate compete with antigens for adsorption sites. Thereupon the surface charges of the adsorbent shifts to values that are unfavorable for adsorption of negative charged antigens and proteins.

The presence of phosphate and citrate leads to desorption of antigens. Therefore, these ions are used at high pH values for identity test of adsorbed vaccines. Ions like chloride and nitrate are adsorbed only weekly. With respect to adsorbed vaccines phosphate and chloride are important, because they are widely used in vaccine formulations and accepted for medical treatment (phosphate buffered media and saline).

Shi, HogenEsch and Hem (2002) studied the ability of intestinal fluid to change the degree of adsorption of ovalbumin to aluminium hydroxide adjuvant or lysozyme to aluminium phosphate. Double distilled water or 0.9% NaCl did not alter the degree of adsorption while citrate at pH 7.4 and phosphate at pH 7.4 reduced the degree of adsorption after a 4 hours exposure period 75 and 45%, respectively. Dilution with sheep lymph fluid reduced the degree of adsorption 25 or 5% after exposure periods of 15 minutes or 4 hours, respectively.

1.3 Aging

Aging or maturation is a well know phenomenon in protein adsorption. The term aging is used here for the observation that protein adsorbates are stabilized by time. During the aging process the adsorbed proteins becomes more inert towards desorption. For model proteins this effect is attributed, e.g. to conformational changes during or after adsorption, to the reorientation of primarily end-on to side-on oriented molecules. For all these cases, the number of contacts between proteins adsorbent is increased during aging, and enthalpic or entropic favourable changes are the driving

forces for these process. In the cases of adsorbed vaccine especially the adsorbed components of diphtheria and tetanus vaccines maturation goes along with an increase in potency. It takes up to several months until equilibrium is reached and a product with constant high potency is formed. This process became important since the introduction of the acellular pertussis vaccines. The lack of the adjuvant effect from the whole cell pertussis components in DTP vaccines leads to a reduced potency with respect to diphtheria and tetanus in DTaP vaccines.

Nail, White and Hem (1976) investigated the aging of aluminium hydroxide gels precipitated at pH 7.0. This process results in larger particles, which were more highly ordered and resistant to attack by acid.

Burrell, White and Hem (2000) tested the stability of aluminium containing adjuvants during aging at room temperature, aluminium phosphate adjuvant and aluminium hydroxide adjuvant had the statistically significant decrease in adsorption capacity which was accompanied by a decrease in surface area.

Shi et al. (2002) studied the ability of intestinal fluid to change the degree of adsorption of ovalbumin to aluminium hydroxide adjuvant or lysozyme to aluminium phosphate. The results of this study indicated that the degree of adsorption upon exposure to intestinal fluid depends on the specific protein and the age of the vaccine.

2. Manufacturing of multicomponent adsorbed vaccines (Matheis et al., 2002)

There are three main strategies for the production of multicomponent adsorbed vaccines (figure 4).

2.1 Sequential adsorption

Sequential adsorption means that first the adsorbent is put in a reaction vessel and the different antigens are added one after the other. In this process the

quality of adsorption depends on the adsorbent, the sequence of antigens adsorbed and the adsorption conditions (e.g. IP, pH and the structure of the antigens). From studies with model systems it is known, that the advantage of a simple procedure goes along with disadvantages regarding complete and safe adsorption. These are

- pre-adsorbed proteins may desorb partly or completely on addition of other proteins;
- later added proteins may not be adsorbed completely;
- proteins may be adsorbed in multiple layers, pre-adsorbed components may be hidden;
- there may be complex changes in surface characteristics of the adsorbent after adsorption of one component that prevent other antigens from being adsorbed (even the adsorption of buffer ions leads to changes in surface charge);
- weakly adsorbed components may be desorbed during storage.

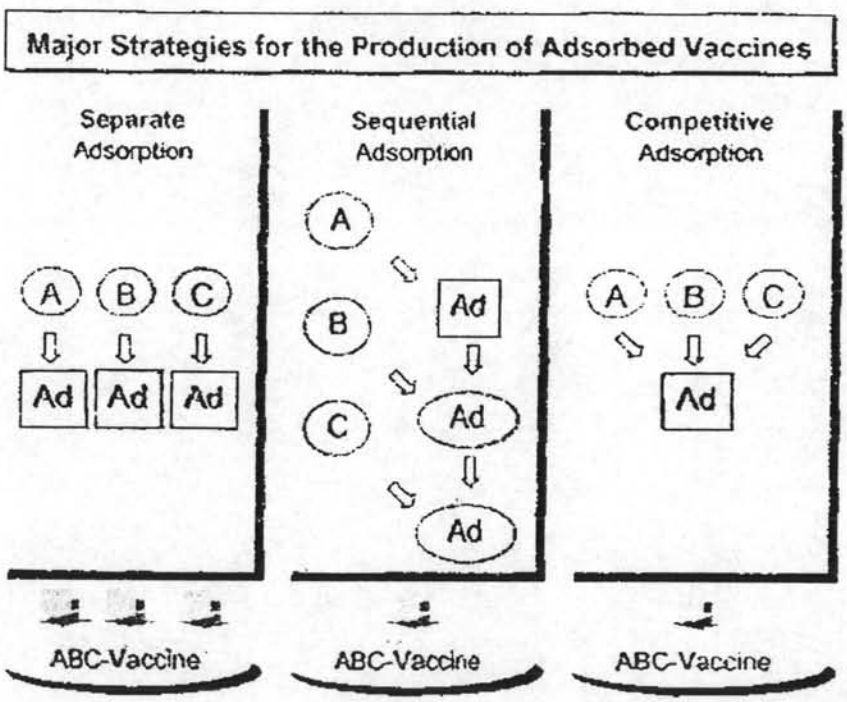

Figure 4. Schematic representation of the three different principle strategies for the production of combined adsorbed vaccines with three different antigens A-C (e.g. DT, TT and PT) adsorbed on an adsorbent (Ad).

2.2 Competitive adsorption

Vaccine production by competitive adsorption means that all antigens are present in the reaction mixture. Formation of the adsorbent and the adsorption of antigens is performed in situ. This strategy of vaccine production is based on early work in vaccine development, when the power of aluminium-containing gels to act as an adjuvant was discovered. In this process the antigens compete for the adsorption sites at the surface of the adsorbent particles. Additionally, it is impossible to control the quality of the gel especially with respect to adsorption capacity and surface characteristics. During development of thermodynamic equilibrium, previously adsorbed antigens may desorb again or some antigens may hide others. Such conditions cannot guarantee shelf life stability, so changes of the final bulk during storage are possible and really observed in some vaccine formulations produced following this procedure. Additionally, the amount of non-adsorbed antigen in the supernatant of these vaccines varied during storage.

2.3 Separate adsorption

Taking into account the above explained theory of adsorption mechanisms, separate adsorption of every antigen is the most likely method for a stable and complete adsorption. Antigens can be adsorbed under controlled and individually optimal conditions. Interfering ions can be separated. If mature adsorbates are used, dilution and mixing for the final formulation will not lead to changes in the degree of adsorption during shelf-life of the vaccine. Even adsorbates of different antigens can be mixed and adjustment of pH to the final value will not lead to detectable changes in the adsorbate composition.

IX. Immunoassay

Immunoanalytical methods, which are based on the binding of small molecules (drugs) or macromolecules by biologically derived antibodies, have revolutionized the field of biomedical analysis. Immunoassays have allowed the

determination of picomolar amounts of analytes that could not be assayed in biological matrices by other techniques. They are therefore, providing information that is essential to the understanding of many biological processes. Since the original work on the analysis of insulin, immunoassay methods have been developed for the determination of a wide variety of drugs, pesticides, hormones, and biological proteins. Immunoassays are not only powerful techniques in terms of sensitivity and specificity but are also relatively simple procedurally. This has led to the development of many kit-type immunoassay systems used routinely in automated clinical laboratories.

The antibodies used as reagents in immunoassays are generally molecules of the immunoglobulin G (IgG) type. These molecules are heterogeneous, bifunctional glycoproteins in which the variable amino acid sequence in the polypeptide component provides its biologic activity. This polypeptide component is made up of two heavy or H chains (50,000 Daltons) and two light or L chains (20,000 Daltons), held together by disulfide bonds. The two binding site of the antibody molecule appear to reside on the NH_2 – terminal ends of the polypeptide chains. They are produced by white blood cells in response to foreign substances introduced into mammalian species. These glycoproteins have the unique property of combining specifically with the substances (antigens) that elicited their formation. This then triggers processes by which the foreign antigens are cleared from the organism, which is the ultimate goal of the immune process.

All immunoassay procedures take advantage of the specific reactions between antibodies and antigens. They involve measurement, directly or indirectly, of the extent of binding between antibodies (reagents) and antigens (analytes). Labels are used in conjunction with the antigens or antibodies in such a way that the concentrations of molecular species can be measured instrumentally. Labels are chemical entities that impart some measurable signal such as radioactivity, fluorescence, or enzyme activity to the antibody or antigen to which it is attached. The extent of antibody binding is determined by measuring the amounts of labeled antigen or antibody in the complexed (bound) and in the free forms. This is generally expressed as the bound/free (b/f) concentration ratio which is related to the

concentration of analyte. The measured signal can be directly or inversely proportional to the b/f ratio, depending on the chemistry of the system (Swarbrick and Boylan, 1993).

1. Enzyme Immunoassay

Quantitation is usually effected by the measurement of spectroscopic properties derived from an enzymatically transformed substrate. The importance of this discovery is reflected in the now widespread application of enzyme-substrate signals for the detection and measurement of soluble antigens, with an attained sensitivity that approaches that of a radioimmunoassay. Today this technology is used in a wide variety of enzyme-based systems both in research and routine analysis. Enzyme immunoassays (EIAs) can be divided into two major classes: homogeneous and heterogeneous immunoassay systems.

1.1 Homogeneous Methods

Homogeneous immunoassay (HOIA) does not require physical separation of the free antigen and antibody-bound antigen because the measured physical signal derived from the antibody-bound, labeled material may by significantly different from that of the unbound entity. There may be an enhancement or an inhibition of enzyme activity upon the binding of Ab to Ag. Homogeneous immunoassay methods are simple to perform and easily automated. Elimination of the separation step avoids a major source of imprecision, but may compromise selectivity since interfering substances are not eliminated in the separation step.

1.1.1 Homogeneous Immunoassays Using Enzyme-Labeled Antigen

Rubenstein et al. described an HOIA method for morphine using lysozyme as the enzyme label. The covalent enzyme-labeled antigen (Ag^E) competes with sample antigen (Ag) for a limited concentration of antibody (Ab) to

form a complex. The resultant complex exhibits very little enzyme activity because of steric hindrance or allosteric inhibition caused by the bound antibody. In the presence of Ag there is competition for the Ab, leaving more Ag^E uncomplexed and free to catalyze the conversion of substrate to product. Thus, the enzyme activity which can be measured by the appearance of product (P) or disappearance of substrate (S) is directly proportional to the amount of free antigen in the sample.

1.1.2 Homogeneous Immunoassays Using an Antigen-Labeled Enzyme Modulator

This method is based on the ability of an antigen labeled with an enzyme modulator (Ag^M) to modulate the activity of an indicator enzyme. The Ag^M competes with free antigen (Ag) for a limited amount of antibody (Ab). On binding with Ab the Ag^M is unable to modulate the activity of the indicator enzyme. As the concentration of the analyte increases, it competes successfully for binding sites on the antibody, leaving more Ag^M free to complex with indicator enzyme, thereby modulating its activity. Based on this principle, practical assays for human serum thyroxine and theophyline have been developed; three distinct classes of modulators have been investigated.

1.1.3 Homogeneous Immunoassay Using an Antigen Labeled with a Fluorogenic Enzyme Substrate

The antigen is linked to a fluorogenic enzyme substrate (Ag^{FS}) to form a stable conjugate, which competes with the sample antigen (Ag) for a limited concentration of antibody (Ab). The antigen-conjugated substrate is a fluorogenic substrate for the enzyme which reacts only when it is not bound to the Ab. Thus, at high concentrations of sample antigen, more of the Ag^{FS} would remain free to act as the substrate for the enzyme (E) and more products would be formed. Thus, fluorescence intensity increases with increasing concentration of the sample antigen. A derivative of umbellifery-β-galactoside serves as a flourogenic substrate for *E. coli* β-galactosidase in this system, and solid-phase reagent strips based on this method have been developed.

1.1.4 Homogeneous Immunoassay Using Liposome - Trapped Enzyme

In this technique, the antigen is labeled with a cytolysin (Ag^{CYT}) capable of lysing a cell membrane or a liposomal membrane. The lytic activity is, however, lost when the conjugate binds to an antibody (Ab) specific for the antigen. The Ag^{CYT} conjugate competes with sample antigen (Ag) for antibody complex formation. An increase in sample antigen concentration, therefore, leads to increased displacement of Ag^{CYT}, leaving the cytolysin conjugate free to lyse the enzyme-containing liposome (L), and thereby releasing more enzyme (E) to form product (P). The enzyme activity is directly proportional to the amount of free sample antigen.

1.2 Heterogeneous Methods

Heterogeneous immunoassays (HEIA) have at least one separation step which allows the differentiation of bound from free material. The enzyme-linked immunosorbent assay (ELISA), is a heterogeneous immunoassay which has been extensively reviewed; either antigen or antibody is immobilized on a solid phase. An essential difference from HOIA is that in HEIA the enzyme label is designed to retain its activity even after its reaction with the antibody. These ELISA typically demonstrate accuracies of 95 to 110% with relative standard deviations (RSD) of 5 to 20%; these values are more than adequate for their intended purpose. The relative lack of control of enzyme-labeling reactions is a limitation of this technique, however, when compared to radiolabeling procedures. In an addition, it is sometimes difficult to purify enzyme-labeled substances.

Heterogeneous methods can be divided into competitive and noncompetitive assays.

1.2.1 Competitive Assays

1.2.1.1 Enzyme-Labeled Antigen Conjugate

The enzyme-labeled antigen (Ag^E) competes with sample antigen for a limited amount of antibody which has been immobilized on a solid phase, for example, polystyrene (Ab^{SP}). After incubation, the unbound Ag^E is separated by washing with a detergent solution. The solid-phase Ab^{SP}, containing bound labeled and unlabeled antigen, is incubated with a substrate (S), and the product concentration is determined with a colorimeter or fluorimeter. The enzyme activity or product concentration is inversely proportional to the concentration of sample antigen. With this competitive method, picogram quantities of hormones and other substances can be measured accurately.

1.2.1.2 Enzyme-Labeled Antibody

This assay employs enzyme-labeled antibody (Ab^E), and the antigen is attached to the solid phase (Ag^{SP}). The binding of Ag^{SP} to Ab^E is competitively decreased by the addition of sample Ag. The enzyme activity is inversely proportional to the concentration of sample Ag. Human IgG at the picomole level has been quantified in less than 1.5 h with this method.

1.2.2 Noncompetitive Assays

1.2.2.1 Enzyme-Labeled Antigen

The sample is first incubated with a moderate excess of solid-phase immobilized antibody (Ab^{SP}). After washing, excess enzyme-labeled antigen (Ag^E) is allowed to bind to unreacted Ab^{SP}. The enzyme product concentration is inversely proportional to the concentration of standard or test antigen.

1.2.2.2 Enzyme-Labeled Antibody

The sample antigen (Ag) is incubated with a moderate excess of enzyme-labeled antibody (Ab^E). The mixture is added to an excess of immobilized antigen (Ab^{SP}) to remove unreacted Ab^E. The enzyme activity is inversely proportional to the concentration of sample and the procedure has been used to measure α-fetoprotein.

1.2.2.3 Sandwich or Double Antibody

This method is used with antigens having multiple antibody-binding sites (epitopes). Immobilized unlabeled antibody (Ab^{SP}), in excess, is incubated with sample or antigen. After washing, the antibody-antigen complex is incubated with an excess of enzyme-labeled antibody (Ab^E) which binds to one or more antigenic sites to form a sandwich-type complex. In this case, the concentration of enzyme product is directly proportional to the concentration of sample antigen.

Sandwich-type assay is well suited for quantifying antigens with multiple antigenic determinants, such as antibodies, rheumatoid factors, a polypeptide hormone, proteins, and hepatitis-B surface antigens. The results obtained are comparable to those obtained with radiolabels in terms of precision, convenience, and sensitivity. Macromolecular antigens at attomole levels have been quantified with this technique.

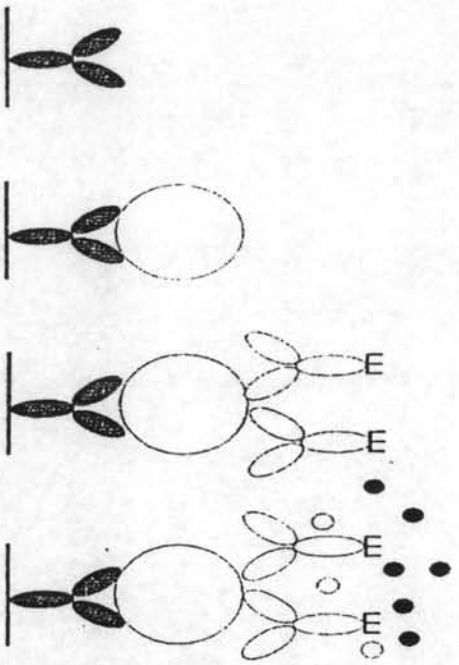

Figure 5 Sandwich ELISA (Crowther, 1996).

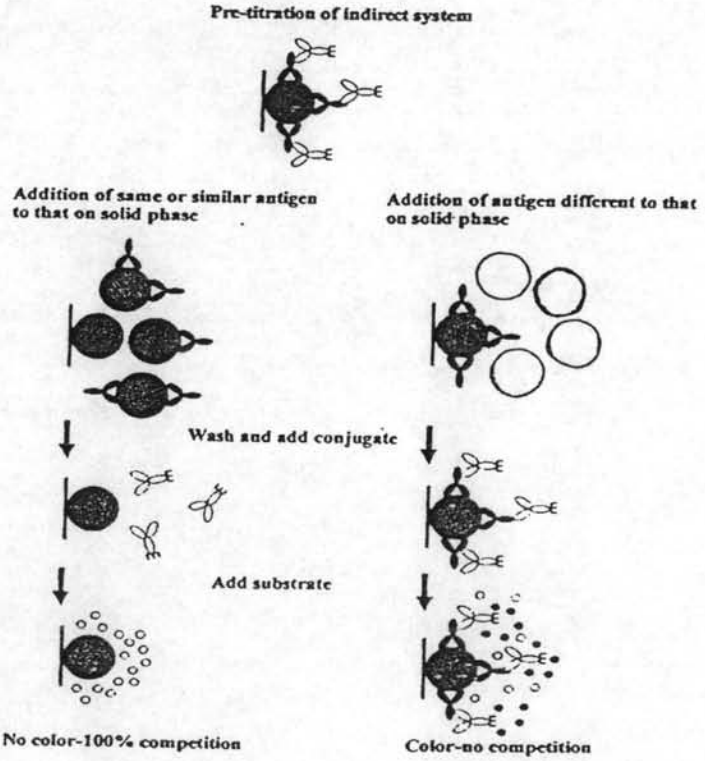

Figure 6 Competition ELISA (Crowther, 1996).