REVIEW OF LITERATURE
Early history

Whooping cough, the most characteristic clinical presentation of *Bordetella pertussis* infection, has been recognised as a distinct entity at least since the 16th century. An account by Guillaume de Baillou or Ballonius (1538-1616) has generally been accepted as the first clear description of epidemic whooping cough. In 1676, Sydenham in England introduced the name "pertussis" meaning a violent cough.

In 1900, Bordet and Gengou observed distinct type of microorganisms in the sputum of a patient with pertussis but it was not until 1906 that they first reported the isolation of the organisms on a potato-glycerol agar (Bordet-Gengou) medium. For many years the bacterium was known as the Bordet-Gengou bacillus and later as *Haemophilus pertussis* (Munoz and Bergman, 1977). In 1952, Moreno-Lopez created a special genus, *Bordetella* to honour Bordet, comprising three serologically related Gram-negative bacilli which have been associated with localized respiratory infection of man and animals. The three species of this genus are *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, of which *B. pertussis* has been the most important member.

Chievitz and Meyer (1916) introduced the cough plate method for bacteriological diagnosis of the disease. Using this method, Kristensen (1933) showed that 75-90% of children expelled *Bordetella pertussis* in air during the catarrhal stage of infection. Maclean (1937) introduced the postnasal or nasopharyngeal swab. Pernasal swab has been found superior to
nasopharyngeal swab (Bradford and Slavin, 1940).

Bordet and Gengou (1906;1907) showed the presence of specific complement fixing antibodies in the blood. This was confirmed by Winholte (1915) and by Chievitz and Meyer (1916). Leslie and Gardner (1931) showed that the organisms might exit in any of the four phases described as phases I, II, III and IV.

**Morphology and cultural characters**

The organisms of *B. pertussis* have been described by Smith (1983) as Gram-negative small coccoid, rod shaped or oval, non motile, non sporing and varying in size from 0.3-0.5 μm X 0.5-1.0 μm. These appear as singles, twos or irregular clumps in smears. Capsules may be seen in young cultures. The organism is strictly aerobic and for primary isolation requires inhibitors of unsaturated fatty acids like blood, charcoal, starch or ion exchange resins. Potato-glycerol-blood-agar medium of Bordet and Gengou is highly suitable.

The organism is slow growing and after 48-72 hours incubation, colonies on Bordet-Gengou medium are small (1 mm in diameter), dome shaped, smooth, opaque, greyish-white, refractile and glistening resembling 'bisected pearls' or 'mercury drops'. Colonies are surrounded by a hazy zone of haemolysis. Optimum temperature for growth is 35°C (Cohen and Wheeler, 1946; Rowatt, 1957) and the optimum pH is 7.4 (Hornibrook, 1939).

*B. pertussis* has no fermentative properties and
biochemically it is inactive. It does not ferment any sugar, nor produces indole, reduces nitrates, utilizes citrate, splits urea or liquifies gelatin. It however, produces oxidase and usually catalase also.

**Antigenic composition and antigenic variation**

*B. pertussis* contains a number of antigens. About 20 different antigens have been demonstrated by gel diffusion (Pusztai, Ekhardt, Jaszovszky and Joó, 1967) and 49 by two dimensional immunoelectrophoresis (Hertz, Holby, Andersen, Wardlaw, Parton, Baek and Hansen, 1979). It was expected that there would be a relationship and perhaps even a crosslinkage between some of the antigens which would be manifested in their serological, protective and/or harmful effects (Pittman, 1979). Bacterial factors thought to be involved in the attachment to the human host are the filamentous haemagglutinin (Sato, Cowell, Sato, Burstyn and Manclark, 1983) and the fimbriae and agglutinogens (Ashworth, Irons and Dowsett, 1982). A number of toxins which have been involved in the pathogenesis include pertussis toxin (Pittman, 1979), adenylate cyclase (Hewlett and Wolff, 1976; Hewlett, Urban, Manclark and Wolff, 1976), dermonecrotic toxin (Livey and Wardlaw, 1984), tracheal cytotoxin (Goldman, Klapper and Baseman, 1982; Goldman and Herwaldt, 1985) and hemolysin (Peppier, 1982). All these factors have been described in detail under the heading "Antigenic and Biologically active components of *B. pertussis*".

Freshly isolated strains of *B. pertussis* are invariably
in a smooth state. Earlier studies indicated that all the recent isolates had a single antigenic type but the laboratory strains were not agglutinated by antiserum raised against fresh isolates (Bordet and Sleeswyk, 1910). Leslie and Gardner (1931) found that fresh isolates were mostly in phase I and laboratory strains in phases II, III and IV. Later Shibley and Hoelscher (1934); Toomey, Ranta, Rohey and McClelland (1935) and Toomey, Takacs and Ranta (1936) observed the phases as not interchangeable states but as stages in the course of Smooth to Rough (S-R) variation.

A number of antigens were found on smooth strains (Flosdorf, Bondi and Dozois, 1941; Flosdorf, Dozois and Kimball, 1941). The 'S' form has been found to be capsulated (Lawson, 1933) but the capsule got easily removed by washing (Miller, 1937; Klieneberger-Noble, 1948). Andersen (1952;1953) recognized five heat labile capsular antigens and a heat stable somatic 'O' antigen. The 'O' antigen has been common to all strains of B. pertussis and cross reactions have been reported to occur with 'O' antigens of B. parapertussis, B. brochiseptica and somatic antigens of 'R' strains of these organisms (Flosdorf et al, 1941; Bondi and Flosdorf, 1943; Eldering, Hornbeck and Baker, 1957).

The 'R' variants of B. pertussis have not been isolated from the human body though they can readily be induced in the laboratory by growing the organisms on an unfavourable medium (Smith, 1983). The 'S' form of the bacterium possessed somatic
antigens associated with virulence. Smith (1983) described various characteristics for phase I strains which included inability to grow on blood agar or nutrient agar, high mouse virulence, high immunizing power in the killed state and presence of haemagglutinin.

The four phase variants I to IV have not been well defined, the characteristics of Phase I and Phase IV could not be described with certainty (Standfast, 1951a; Standfast, 1951b; Parker, 1979). Phase IV strains were devoid of the mouse virulence, protective antigen, histamine sensitizing factor and agglutinogens (Kind, 1953; Aprile, 1972). Parker (1976;1979) regarded phase variation as the result of loss mutation in a number of different genes independently in response to the selection pressure of the artificial culture.

Apart from loss variation, B. pertussis was also observed to undergo a rapid, phenotypic change called modulation (Lacey, 1960). When the organism was cultured in presence of magnesium ions in place of sodium ions or at low temperature (Lacey, 1960) or at a high concentration of nicotinic acid (Pusztai and Jóó, 1967). the typical strains termed as 'X-mode' became 'C-mode'. The 'C-mode' organisms lacked in hemolytic activity, haemagglutinating activity, mouse protective antigen, histamine sensitizing and lymphocytosis promoting factors (Lacey, 1960; Holt and Spasojevic, 1968; Parton and Wardlaw, 1975; Wardlaw, Parton and Hooker, 1976). The intra and extra cellular adenylate cyclase activity was also lost in 'C-mode' as well as in Phase IV
organisms (Parton and Durham, 1978). Although the Phase IV and 'C-mode' organisms appeared similar in various characteristics, the two terms should not be regarded as interchangeable, phase variation being a loss variation and modulation the result of phenotypic change (Smith, 1983).

**Antigenic and Biologically active components of B. pertussis**

Several antigenic and biologically active components have been demonstrated in *B. pertussis* which have been very well reviewed in a number of articles (Pittman, 1970; Munoz and Bergman, 1977; Pittman, 1979; Munoz and Bergman, 1979; Wardlaw and Parton, 1983a, b; Pittman, 1984b; Manclark and Cowell, 1984; Robinson et al, 1985). The various components are:

1. Filamentous haemagglutinin (FHA)
2. Agglutinogens and fimbriae
3. Dermonecrotic toxin
4. Lipopolysaccharide (Endotoxin)
5. Adenylate cyclase
6. Tracheal cytotoxin
7. Hemolysin
8. Pertussis toxin or Pertussigen (Histamine sensitizing factor (HSF), Lymphocytosis/Leucocytosis promoting factor (LPF), Islet activating protein (IAP), mouse protective antigen, late appearing toxicity factor, heat labile adjuvant factor)
1. Filamentous haemagglutinin

Haemagglutinin (HA), a substance derived from *B. pertussis* with the ability to directly agglutinate erythrocytes from chickens and other animals was first described by Keogh, North and Warburton (1947) and Keogh and North (1948). HA was mainly found in cells of very young cultures grown on solid or in liquid media. As liquid grown cultures grew older, most of the HA was found in the cell-free supernatant fluid.

The HA agglutinated human O, A, B, and AB erythrocytes as well as chicken, mouse, guinea pig, rabbit, sheep and horse red blood cells, while ox erythrocytes were not agglutinated unless pretreated with trypsin (Munoz and Bergman, 1977). HA bound to different receptors than those reacting with influenza virus (Fisher, 1948).

Keogh and North (1948) and Ungar (1949) found HA associated with virulence but Standfast (1951a) could not confirm this finding. Keogh and North (1948) reported that HA may be protective to mice and children but Pillmer (1950); Thiele (1950) and Masry (1952) did not agree with this. However, Sato, Arai and Suzuki (1973); Morse and Morse (1976) and Arai and Sato (1976) isolated HA and found that it protected mice from infection.

Arai and Sato (1976) and Irons and MacLennan (1979a, b) reported two distinct haemagglutinins from Phase I strains of *B. pertussis*. The lymphocytosis promoting factor hemagglutinin (LPF-HA) appeared as spherical structures, 6 nm in diameter by electron microscopy (Morse and Morse, 1976; Askelöf and
Gillenius, 1982), had a relatively low haemagglutinating activity and induced a variety of biological responses. The filamentous haemagglutinin (FHA) appeared as fine filaments about 2 nm in diameter and 40 to 100 nm in length (Arai and Sato, 1976; Morse and Morse, 1976) and had HA activity per mg of protein 5 to 7 times greater than that of LPF-HA (Arai and Sato, 1976; Irons and MacLennan, 1979a).

FHA has been purified mostly from static liquid culture supernatants (Arai and Sato, 1976; Irons and MacLennan, 1979a,b; Arai and Munoz, 1979a,b; Ashworth et al, 1982; Selmer, Larsen, Hertz and Parton, 1984) and as well as from the extracts of cells grown on solid medium (Nakase, Doi and Kasuga, 1975; Askelöf, Granström, Gillenius and Lindberg, 1982; Irons, Ashworth and Wilton-Smith, 1983). A pure preparation of FHA free from LPF has been obtained by sequential steps of hydroxylapatite, sepharose-6B gel filtration and affinity chromatography (Cowell, Sato, Sato, Lan and Manclark, 1982; Sato et al, 1983). FHA has been found to be a protein with a molecular weight of 130,000 (Sato et al, 1983). Several workers have found FHA to be heterogenous with major polypeptides of molecular weights of 90,000 to 220,000 (Arai and Sato, 1976; Irons and MacLennan, 1979b; Robinson, Hawkins and Irons, 1981; Cowell et al, 1982; Irons et al, 1983). The activity of FHA has been inhibited by small concentrations of cholesterol (Irons and MacLennan, 1979a,b; Sato et al, 1983).

Previously, it was considered that FHA was derived from
fimbriae (Sato, Arai and Suzuki, 1974; Morse and Morse, 1976; Sato, Izumiya, Oda and Sato, 1979). Recently, Ashworth et al. (1982); Ashworth, Dowsett, Irons and Robinson (1985) and Zhang, Cowell, Steven and Manclark (1985) concluded that FHA has not been derived from fimbriae. However, the role of FHA in mediating attachment of the organism to ciliated cells has great significance (Sato et al., 1979; Sato, Izumiya, Sato, Cowell and Manclark, 1981; Tuomanen, Weiss, Rich, Zak and Zak, 1985; Urisu, Cowell and Manclark, 1985; Tuomanen and Weiss, 1985). The protective role of FHA in animals (Ashworth, Fitzgeorge, Irons, Morgan and Robinson, 1982; Cowell, Oda, Burstyn and Manclark, 1984) and its low toxicity (Robinson et al. 1985; Redhead and Seagroatt, 1986) make it one of the possible components for the acellular pertussis vaccine (Manclark and Burns, 1985). In the Japanese acellular pertussis vaccine, FHA has been included as one of the components (Sato et al., 1984). Recently FHA gene and gene for the B. pertussis outer membrane protein have been cloned in Escherichia coli (Reiser, Friedman and Germanier, 1985; Shareck and Cameron, 1985).

2. Agglutinogens and fimbriae

The term "agglutinogen" is given to the substance or substances that react with their corresponding antibodies and cause the agglutination of the cells on which they are present. The antigenic complexity of the agglutinogens of B. pertussis was not fully realized until Andersen (1953) and Eldering, Hornbeck and Baker (1957) showed that smooth strains had a
common heat stable 'O' antigen and one or more thermolabile 'K' antigens. Eight different agglutinogens have been functionally described for \textit{B. pertussis}. Six (agglutinogens 1-6) are species specific and two (agglutinogens 7 and 13) are shared by other species of the genus \textit{Bordetella}. Agglutinogen 1 is common to all strains of \textit{B. pertussis}, whereas agglutinogens 2-6 have been found in various combinations as strain specific. Agglutinogen 2, 3 and 5 resisted heating at 100°C for 2½ hours but 1, 6 and 7 were destroyed by this treatment (Eldering, 1962).

Agglutinogen as purified by Onoue, Kitagawa and Yamamura (1961) was antigenic and produced specific skin reactions of induration and erythema in sensitized rabbits. Agglutinogen preparations have been used as skin test reagents to detect susceptibility to whooping cough in children and to evaluate the efficacy of vaccines. The positive skin test correlated with serum agglutinins (Flosdorf, Felton, Bondi and McGuiness, 1943).

A relationship has been found between agglutinins in children and their resistance to whooping cough (Medical Research Council, 1959) but this is to be expected only when complex vaccines are used since the children would develop antibodies against the various antigens found in the vaccine. Purified agglutinogen (factor 1) has not been effective in protecting mice from intracerebral challenge (Schuchardt, Munoz, Verwey and Sagin, 1963; Ross and Munoz, 1971). Preston (1963; 1966) and Preston and Stanbridge (1972) have emphasized the role of agglutinogens in inducing protection in children. Stanbridge
and Preston (1974) developed a marmoset protective test which has been claimed to be specific for agglutinogen factors and could prove to be of value in evaluating the potency of vaccines. Evans and Perkins (1953; 1954) reported that agglutinin production in mice was graded in response to inoculation of graded doses of pertussis vaccine but Saran, Angra, Sahai and Balasubrahmanyan (1979) and Saran, Kaushik and Balasubrahmanyan (1981) did not agree with this. Agarwal and Preston (1976) found the mouse to be unsuitable animal to estimate the agglutinogen content of pertussis vaccine by agglutinin production.

Recently agglutinogens 2 and 3 have been purified and their fimbrial nature has been reported (Ashworth et al. 1982; Carter and Preston, 1984; Fredriksen, Froholm and Paulsen, 1985; Robinson et al., 1985; Ashworth et al., 1985; Irons, Ashworth and Robinson, 1985; Zhang et al., 1985). However, Preston (1985) maintained that agglutinogen 3 is non fimbriate. The protective nature of agglutinogens in animals (Robinson, Ashworth, Baskerville and Irons, 1985; Zhang et al., 1985) and the proven involvement of fimbriae in adherence of bacteria to mammalian cells (Robinson et al., 1985 and Ashworth, Robinson, Irons, Dowsett, Gorringe and Wilton-Smith, 1986) make the agglutinogens one of the prospective components of acellular pertussis vaccine (Manclark and Burns, 1985). The British scientists have been considering to include the agglutinogens as one of the components in their acellular pertussis vaccine (Miller, 1986).
3. Dermonecrotic toxin

The dermonecrotic toxin or heat labile toxin was first described by Bordet and Gengou (1909). It has been found lethal to guinea pigs, rabbits or mice when given intraperitoneally or intravenously. The toxin was dermonecrotic and got inactivated at 56°C in 10 minutes (Evans and Maitland, 1937; Munoz, Ribi and Larson, 1959; Munoz, 1971). The suckling mouse provided the most sensitive model of detecting necrotic activity (Katsampes, Brooks and Bradford, 1942). Inspite of the dramatic demonstrations with animals, the role of the toxin in human infection has not been clearly defined (Pittman, 1970). Doubt about its role arose from the lack of antitoxin in convalescent serum (Anderson and North, 1943; Evans, 1947) whereas the extract of bacteria induced antitoxin. It produced a pronounced degeneration of the spleen (Iida and Okonogi, 1971; Munoz and Bergman, 1977; Sekiya, Munoz and Nakase, 1982; Sekiya and Munoz, 1982; Sekiya and Munoz, 1983) and possibly lymph nodes (Munoz, 1971). The spleen size of mice also decreased after lethal intracerebral challenge (Berenbaum, Ungar and Stevens, 1960).

Recently Nakase and Endoh (1985) and Endoh, Amitani and Nakase (1986) reported purification of this toxin and characterized it as a protein with molecular weight of 102,000. The suppression of antibody production by the dermonecrotic toxin observed by Sekiya (1983) has been considered to be the result of splenic insufficiency due to vasoconstrictive activity and not by the direct action of the toxin on the
antibody producing cells (Nakase and Endoh, 1985). As the
dermonecrotic toxin has been claimed to cause many of the
symptoms of local disease, it may be considered for inclusion
in the second generation acellular pertussis vaccines after
toxoiding the toxin (Manclark and Burns, 1985).

4. Lipopolysaccharide (Endotoxin)

The heat stable endotoxin obtained from the cell walls of
*B. pertussis* has been similar to the endotoxins isolated from
other Gram-negative bacteria (Munoz, 1963). The lipopolysaccharide
of *B. pertussis* had certain distinct chemical properties also
(Wardlaw and Parton, 1983b). The preparations are pyrogenic and
may increase susceptibility of experimental animals to infection
or provide non-specific protection against infection, have
adjuvant properties and lack protective activity *per se* (Pittman,
1957; Kind, 1958; Munoz, 1963; Sutherland, 1963). The toxin
resists heating at 100°C for one hour.

The endotoxin is antigenic and sera prepared in animals
against whole cells or extracts contained easily demonstrable
precipitins to endotoxin (Ashworth, *et al.*, 1982). The antibodies
have also been detected in humans after vaccination with whole
cell pertussis vaccine or after infection with *B. pertussis*
(Ashworth, Robinson, Irons, Morgan and Isaacs, 1983). It is a
good adjuvant to stimulate antibodies to various antigens
(Sultzzer, Craig and Castagna, 1985; Winters, Baggett, Lee, Sloan.
Lemmon and Stinson, 1985) but it is not the only adjuvant in
*B. pertussis* (Morse, 1976).
The lipopolysaccharide is mainly responsible for most of the early side effects of whole cell pertussis vaccine including fever and this toxin has been associated with the early weight loss in the mouse weight gain test (Manclark and Cowell, 1984; Redhead and Seagroatt, 1986). In the Japanese acellular pertussis vaccine the endotoxin has been completely removed (Sato et al., 1984) as it did not have any role in protection against whooping cough (Manclark and Burns, 1985).

5. Adenylate cyclase

Bacterial adenylate cyclase, converting adenosine triphosphate (ATP) to adenosine cyclic 3', 5' monophosphate (cyclic AMP), has been reported to be extracytoplasmic in _B. pertussis_ (Hewlett and Wolff, 1976; Hewlett et al., 1976). The extracytoplasmic adenylate cyclase activity of _B. pertussis_ has been in addition to the adenylate cyclase activity of pertussis toxin (Manclark and Cowell, 1984). The former activity has been due to a monomeric protein with a molecular weight of 70,000 (Hewlett and Wolff, 1976). This enzyme is heat stable and is regulated by eukaryotic regulatory protein calmodulin (Wolff, Cook, Goldhammer and Berkowitz, 1980; Goldhammer, Wolff, Cook, Berkowitz, Klee, Manclark and Hewlett, 1981). The enzyme has been detected in the culture supernatants (Endoh, Takezawa, and Nakase, 1980) and has been purified recently (Hewlett, Weiss, Crane, Pearson, Anderson, Myres, Evans, Hantske, Kay and Cronin, 1985). This enzyme behaved like toxin conforming to the A/B model for bacterial toxins (Hewlett et al., 1985). Confer
and Eaton (1982 and 1985) reported that the adenylate cyclase entered the phagocytic cells contributing to the pathogenicity of *B. pertussis* by inhibiting the macrophages and even lymphocytes (Hewlett, Smith, Myres, Pearson and Kay, 1983; Hewlett, Weiss, Pearson, Myres and Cronin, 1985). Adenylate cyclase has been found to be one of the important virulent factors (Weiss, Hewlett, Myres and Falkow, 1985; Wolff, 1985) and protected mice against intracerebral challenge (Novotny, Chubo, Cownley, Montaraz and Beesley, 1985). Due to these properties of adenylate cyclase, it has been considered to be included as one of the prospective antigens for the acellular pertussis vaccine (Manclark and Burns, 1985; Novotny et al., 1985).

6. Tracheal cytotoxin

Tracheal cytotoxin isolated and purified by Goldman et al. (1982) is a glycopeptide which is released during log phase broth culture. Further purification and characterization of tracheal cytotoxin has been reported by Goldman and Herwaldt (1985). Its *in vitro* action on the hamster tracheal organ cultures resembled the typical pathological feature of pertussis. No other toxin or component from *B. pertussis* has been able to produce such effect i.e. the loss of ciliated cells in the respiratory epithelium (Goldman and Herwaldt, 1985). The toxin inhibited DNA synthesis in a cell culture of hamster tracheal epithelial cells and caused cellular damage and ciliostasis to hamster tracheal epithelial cells (Goldman et al., 1982). Tracheal cytotoxin has been considered as one of the prospective antigens to be included for the second generation acellular vaccines.
after its safety and protection is confirmed in laboratory animals (Manclark and Burns, 1985).

7. Hemolysin

Hemolysin, another component of *B. pertussis* reported by Peppler (1982) has not yet been purified. However, it has been considered as one of the important virulence factors (Weiss et al., 1985). Parker, Branes, Armstrong, Frank and Cole (1985) suggested that hemolysis, adenylate cyclase activity, dermo-necrotic toxic activity and 24 hour toxicity were associated with a single component— a vesicular package of toxins and adhesins. The production of hemolysin has been reported by the Phase I cultures of *B. pertussis*, similar to the production of other biologically active components (Goldman, Hanski and Fish, 1985).

8. Pertussis toxin or Pertussigen

The term "Pertussis Toxin" has been proposed by Pittman (1979) for a component of *B. pertussis* organisms having several biological activities. Previously, various names based on the biological activities of the factor were given to the pertussis toxin. These included the histamine sensitizing factor (HSF) (Parfentjev and Goodline, 1948), the leucocytosis/lymphocytosis promoting factor (LPF) (Morse, 1965), late appearing toxicity factor (Kurokawa, Iwasa and Ishida, 1965), the islets activating protein (IAP) (Yajima, Hosoda, Kanbayashi, Nakamura, Nogimori, Mizushima, Nakase and Ui, 1978) and pertussigen (Munoz, 1976). The LPF-HA described by Cowell et al. (1982) and Sato, Sato,
Izumiya, Cowell and Manclark (1982) had the same activities as shown by the pertussis toxin. A number of reviews have described the purification and biological activities of this toxin in detail (Pittman, 1970; Munoz, 1971; Munoz and Bergman, 1977; Munoz, Bergman, Cole and Ayers, 1978; Pittman, 1979; Munoz and Bergman, 1979; Munoz and Arai, 1982; Wardlaw and Parton, 1983a,b; Manclark and Cowell, 1984; Sekura, Zhang and Quentin-Millet, 1985; Munoz, 1985; Robinson et al, 1985; U1, Nogimori and Tamura, 1985; Pittman, 1986).

Pertussis toxin or HSF has been shown to be located in the cell wall or closely associated therewith (Munoz et al, 1959; Billaudelle, Edebo, Hammersten, Heden, Malmgren and Palmstierno, 1960; Munoz and Bergman, 1968). It has been found to be thermostable at 80°C for 30 minutes (Maitland, Kohn and McDonald, 1955; Kind, 1956) and is a globular protein with a molecular weight of 117,000 having five polypeptide chains named as S1, S2, S3, S4 and S5 (Tamura, Nogimori, Murai, Yajima, Ito, Katada, U1 and Ishii, 1982; Peppler, Judd and Munoz, 1985). The toxin was a hexamer containing 2 subunits of the S4 peptide and one each of S1, S2, S3 and S5 peptides. The composition of toxin has been described as an A-protomer and a B-oligomer and the mechanisms of actions of these two subunits have recently been described (Tamura, Nogimori, Yajima, Ase and U1, 1983; Nogimori, Tamura, Nakamura, Yajima, Ito and U1, 1985; U1, Nogimori and Tamura, 1985). The B-oligomer bound to the cell surface producing many biological activities and the A-protomer was transported across the plasma membrane to its target sites within the cells.
The HA activity of pertussis toxin has been due to B-oligomer (Manclark and Cowell, 1984) which is inhibited by haptoglobin and fetuin unlike that of FHA which was inhibited by cholesterol (Irons and MacLennan, 1979a,b). Several methods of purification of pertussis toxin have been reported even on commercial scale for the production of acellular pertussis vaccine (Yajima et al, 1978; Irons and MacLennan, 1979a,b; Arai and Munoz, 1981; Munoz and Arai, 1982; Cowell et al, 1982; Askelöf and Gillenius, 1982; Sato et al, 1983; Sekura, Fish, Manclark, Meade and Zhang, 1983; Sato et al, 1984; Munoz, 1985; Roumiantzeff et al, 1986; Trollfors, 1986).

In mice, the toxin induced physiologic and pharmacologic changes leading to an increased sensitivity of these animals to many types of shock such as anaphylactic, histamine, serotonin, cold, X-ray, endotoxin, anoxia etc. (Munoz and Bergman, 1977; Munoz, 1985). Furthermore, it induced lymphocytosis and acted as an immunological adjuvant when given with an antigen increasing production of antibodies of various classes including the Ig E class of immunoglobulins in rats as well as in mice (Wardlaw, Parton, Bergman and Munoz, 1979; Sadowski, Robbins and Munoz, 1979; Iwata, Huff, Uede, Munoz and Ishizaka, 1983; Mitchell and Munoz, 1983; Sekiya, 1983). Pertussis toxin also accelerated induction of experimental allergic encephalomyelitis in rats and mice receiving encephalitogenic antigen (Levine, Wenk, Devlin, Pieroni and Levine, 1966; Bergman and Munoz, 1976; Bergman, Munoz and Portis, 1978; Linthicum, Munoz and Blaskett, 1982; Waxman, Bergman and Munoz, 1982; Munoz, 1983; Munoz and
Mackay, 1984a, b; Munoz, Bernard and Mackay, 1984) and protected mice from intracerebral challenge with virulent *B. pertussis* (Sato *et al.* 1974; Cowell *et al.* 1982; Sato and Sato, 1985; Manclark and Burns, 1985; Robinson *et al.* 1985; Munoz, 1985). LPF or pertussis toxin inhibited the *in vitro* chemotactic response of murine macrophages and affected other cells of immune system (Andersen, Hertz, Sorensen, Baekgaard, Christensen, Ramhoj, Hansen, Wardlaw and Sato, 1977; Sugimoto, Nakanishi, Otokawa, Uchida, Yasuda, Sato and Sato, 1983; Meade, Kind, Ewell, McGrath and Manclark, 1984; Becker, Kermode, Naccache, Yassin, Marsh, Munoz and Sha'afi, 1985; Shefcyk, Yassin, Volpi, Molski, Naccache, Munoz, Becker, Feinstein and Sha'afi, 1985; Volpi, Naccache, Molski, Shefcyk, Huang, Marsh, Munoz, Becker and Sha'afi, 1985). Delayed hypersensitivity induced by whole cell pertussis vaccine or pertussigen has been demonstrated which could be transferred with lymph nodes but not with serum to normal animals (Rowley, Chutkow and Attig, 1959; Gruenewald, Allen, Levine and Wonk, 1961; Sewell, Munoz and Vadas, 1983; Tamura, Nakamishi, Kojima, Otokawa, Uchida, Sato and Sato, 1983; Gamble, Vadas, Munoz, Thomas and Miller, 1983; Sewell, Munoz, Scollag and Vadas, 1984).

Various hypotheses have been put forward to explain the pertussis enhanced hypersensitivity (Munoz and Bergman, 1979). Fishel, Szentivanyi and Talmage (1962) reported that it was due to the functional imbalance between two types of adrenergic receptors or in the neural pathways leading to them. Subsequent work indicated that HSF blocked the β adrenergic system.
functionally associated with the inhibition of muscle uptake of glucose (Fishel, szentivanyi and Talmage, 1964). It has been proposed that vaccine through β adrenergic blockade induced hypoglycemia which mediated a convulsive state in the child leading to further neurological complications (Pittman, 1970). During whooping cough, the blood sugar level dropped below normal (Regan and Tolstoouhov, 1936).

Recently the cloning of pertussis toxin gene has been reported (Locht, Barstad, Coligan, Mayer, Munoz, Smith and Keith, 1986). Several properties of LPF or pertussis toxin including its induction of clustered growth of Chinese hamster ovary cells (Hewlett, Sauer, Myers, Cowell and Guérant, 1983) and its binding with fetuin and haptoglobin have been used for assaying the toxin and antibodies against it (Irons and MacLennan, 1979b; Askelöf et al., 1982; Sato, Sato and Ito, 1983; Sekura et al., 1983; Gillenius, Jaatmaa, Askelöf, Granström and Tiru, 1985).

The reactogenicity of pertussis vaccine has been mainly due to the pertussis toxin and attention has been given now to methods for its toxicing (Manclark and Cowell, 1984). Estimation of its biological activities like HSF have been correlated with reactions in children (Conen, 1968; Perkins, Sheffield, Miller and Skegg, 1970). Detoxification of pertussis toxin in pure form or alongwith the whole cells with formaldehyde and glutaraldehyde has resulted in the destruction of its various biological activities and toxicity to variable degrees with some effects on intracerebral mouse protective activity (Munoz and Hestøkin.
1966; Sato et al. 1979; Munoz, Arai and Cole, 1981; Munoz, Arai, Bergman and Sadowski, 1981; Sato et al. 1982; Munoz and Arai, 1982; Cowell et al. 1982; Robinson and Irons, 1983; Relyveld and Ben-Efraim, 1983; Manclark and Cowell, 1984; Robinson et al., 1985; Gupta et al., 1987a,c,d). The pertussis toxin after toxoiding has been considered as one of the primary and most important component of acellular pertussis vaccine (Manclark and Burns, 1985). This component has been included in the Japanese acellular pertussis vaccine and is under active consideration to be included as the only component in French and American acellular vaccines (Roumiantzeff et al., 1986; Trollfors, 1986). Manclark and Cowell (1984) cautioned that the use of vaccine that prevented some of the important symptoms of disease but did not prevent infection might constitute an important reservoir of disease and a threat to unimmunized individuals.

Pathogenesis

Pertussis is a disease of man but experimental infection has been produced in several species of animals like guinea pig (Lesile and Gardner, 1931), monkey and rabbit (Witebsky and Salm, 1937; Sprunt, Martin and McDearman, 1938), mouse (Burnet and Timmins, 1937; Bradford, 1938), chick embryo (Gallavan and Goodpasture, 1937) and sheep (Standfast, 1958).

Intranasal inoculation of *B. pertussis* culture in mice produces patchy interstitial pneumonia, histologically resembling the human disease. Large doses given intraperitoneally were fatal due to txaemia while intracerebral inoculation
caused a fatal infection (Pittman, 1970). Immunized mice are protected against intracerebral inoculation forming the basis of intracerebral mouse potency assay for pertussis vaccine (Kendrick, Eldering, Dixon and Misner, 1947).

*B. pertussis* is not invasive (Trollfors, 1984). In man the typical disease starts by the attachment of the organisms to ciliated cells in the trachea and bronchi (Linnemann, 1979). The disease includes three stages after an incubation period of 7-14 days: catarrhal, paroxysmal and convalescent (Lapin, 1943; Court, Jackson and Knox, 1953). The catarrhal stage lasting 1 to 2 weeks begins with nonspecific respiratory symptoms, malaise, anorexia and sometimes a low grade fever and ends with increasingly severe cough. The paroxysmal stage lasting several weeks begins with paroxysmal coughing. During this stage the characteristic "whoop" appears which is produced when prolonged coughing is followed by forced inspiration over a partially closed glottis (Linnemann, 1979). The paroxysms of coughing may be followed by vomiting and may be associated with epistaxis, conjunctival or scleral hemorrhages and lymphocytosis which appear late in the catarrhal stage and continue to increase in the paroxysmal stage (Thelander, Henderson and Kligariff, 1933). Maximum lymphocytosis corresponded to the time of most severe coughing and persisted for 2-3 months (Lapin, 1943). Leucocytosis occurred also in mice inoculated intranasally with the organisms (Cooper, 1952). The sedimentation rate was normal or decreased and some patients developed hypoglycemia (Regan and Tolstoouhov, 1936; Lagergren, 1963).
During the final or convalescent stage sporadic coughing may continue for weeks to months. Complications like subconjuctival haemorrhage, emphysema, bronchopneumonia, lung collapse, convulsions, coma and encephalopathy may arise due to infection. Respiratory complications are self limited, the atelectasis resolving spontaneously but the neurological complications may result in permanent sequelae such as epilepsy, paralysis, growth retardation, blindness and deafness (Linnemann, 1979).

Recently it was shown that the pertussis has been a toxin mediated disease (Pittman, 1984b; Pittman, 1986). The pertussis toxin, an exotoxin was liberated by the organisms in situ when these were present on cilia (Pittman, 1984b).

Epidemiology

Man has been the only known reservoir of pertussis and infection is transmitted by symptomatic patients (Trollfors, 1984). There have been no proven chronic carriers of B. pertussis (Linnemann, Bass and Smith, 1968) but a few subclinical infections have been detected by culture in healthy home contacts of patients (Manclark and Cowell, 1984). The source of infection is the catarrhal stage of the disease. Infection is transmitted by droplets and by fomites contaminated with oropharyngeal secretions (Mueller et al, 1986). Whooping cough is one of the most infectious bacterial diseases and non immune contacts seldom escape the disease.

The disease is worldwide in distribution. It occurs as
epidemics periodically but the disease is never absent from any community. In England, pertussis epidemic occurred at 4 years interval (Preston, 1984a). The disease was more common in female children than the male ones (Cruickshank, Duguid, Marmion and Swain, 1976; Mueller et al, 1986).

In the beginning of the twentieth century, whooping cough in Britain and other Western countries killed some 10,000 children annually (Wilson and Miles, 1975). Recently morbidity and mortality have fallen progressively as a result of the availability of effective vaccine and antibiotics. The World Health Organization estimated 60 million cases of pertussis annually with half a million to one million deaths (Mueller et al, 1986). Most of these cases occurred in developing countries where immunization coverage is low. Since maternal antibody did not seem to protect against the disease, there is a need to vaccinate the children at an early age. The disease may occur in any season during the year but the incidence in Western countries has been higher in late winter and spring (Wilson and Miles, 1975).

In India whooping cough is endemic throughout the year but occasionally present in an epidemic form. Dayal, Atal and Kumar (1969), in a study of 230 clinically diagnosed cases of whooping cough isolated B. pertussis in 63.36% of children between 3-5 years of age. Maximum incidence was observed during February, March and April. Chavan (1971) found the maximum incidence during December, March and April in a study at Bombay involving 516 suspected cases of whooping cough. Ray, Agarwal
and Chitkara (1973) isolated *B. pertussis* from 4 cases (7.7%) out of 52 children aged 1½ months to 11 years with clinical symptoms of whooping cough from Chandigarh.

Agarwal, Ray, Chitkara and Walia (1975) found 22% of healthy unvaccinated children showing agglutinin titre of equal to or more than 1:20 while only 7 (12.6%) out of 51 suspected cases were proven cases of pertussis on the basis of bacteriological and/or serological evidence. Saran, Angra and Balasubrahmanyan (1979) found 47.71% of the cases having positive agglutinin titre for *B. pertussis* and 70.21% for *B. parapertussis* in apparently normal persons aged between 5-55 years. Ray, Grover and Rai Chowdhuri (1984) found 16% of children below 6 months of age having agglutinins, while after 6 months, rise to 39% was seen which gradually increased to 48% at 2 years of age and 60% at 2-4 years of age.

The occurrence of pertussis cases ranged from 150,652 to 216,548 per year during 1968-1972 as reported by Directorate General of Health Services (D'Sa, 1974). These figures do not seem to be complete and incidence of the disease could be higher (D'Sa, 1974). According to E.P.I. Bulletin (1981), the reported number of cases ranged from 212,154 to 483,415 per year during 1972-1980 with 101 to 473 deaths per year during this period. The prevalent serotypes of *B. pertussis* in India have been 1 and 3, as also found in United Kingdom and other countries (D'Sa, 1974).
Prophylactic vaccination against pertussis

The use of vaccine against pertussis seems to have been started in 1909 by Freeman. In 1913, Nicolle and Conor used a live pertussis vaccine during an outbreak of the disease in Tunis. Killed vaccines have been used for the immunization of children by Huenekens (1917) and Hewlett (1929).

The first trials with the vaccine indicated that the use of a vaccine prepared from a freshly isolated strains of B. pertussis was chiefly of value in decreasing the severity and mortality of the disease (Madsen, 1933). In a number of trials in the United States, a substantial lowering of the attack rate was observed in children injected with plain or alum-precipitated vaccine (Sauer, 1937; Kendrick and Eldering, 1936,1939; Kendrick, 1942,1943; Bell, 1941,1948). Further favourable reports were made by a number of workers in other countries (Rambar, Howell, Donenholz, Goldman and Stanard, 1941; Perkins, Stebbins, Silverman, Lembcke and Blum, 1942; Oláh, 1942; Dungal, Thoroddsen and Ágústsson, 1944). But Siegel and Goldberger (1937) and Doull, Shibley, Haskin, Bancroft, McClelland and Hoelscher (1939) in the United States and McFarlane, Topley and Fisher (1945) in England failed to obtain any substantial degree of protection in controlled field trials among infants and children.

The British field trials conducted in 1950s by Medical Research Council concluded that it was possible by vaccination to produce a high degree of protection against the disease, as shown by substantial reduction in the attack rates among the
home contacts and reduction in the severity and duration of the disease where the vaccination failed to give complete protection. The results also showed that the different vaccines employed varied a great deal in their protective action: the low potency vaccines gave an attack rate in home contacts of 87% and the most potent vaccines gave an attack rate of 4% only (Medical Research Council report, 1959). The risks and benefits of pertussis immunization have been still controversial (Editorials, 1981). Miller, Alderslade and Ross (1982) concluded that the disease remained too dangerous in relation to the known hazards of the vaccine and recommended the continuation of vaccination.

The pertussis vaccine is generally given as adsorbed vaccine along with diphtheria and tetanus toxoids in the form of adsorbed D.P.T. vaccine, and also with inactivated polio vaccine in the form of quadruple vaccine (Cohen and Nagel, 1984; Swartz, Roumiantzeff, Peyron, Stopler, Kanaaneh, Leitner and Goldblum, 1985; Drucker, Schatsmayr, Grenier, Ajjan, Peyron and Roumiantzeff, 1985). Methods of production of pertussis vaccine differ between manufacturers. Even an individual manufacturer's product shows some variation from lot to lot. The method for the production of pertussis vaccine of acceptable quality meeting the W.H.O. requirements has been described by the World Health Organization (W.H.O., 1977).

The whole cell vaccine can be produced in liquid or solid media. The strains of B. pertussis to be used for vaccine production should include agglutinogens 1, 2 and 3 (W.H.O.,
1979). The inactivation of the organisms can be done by heating at 37°C or 56°C or by short exposure to formalin or by 0.02% merthiolate (Pittman, 1970). Other factors that vary between laboratories have been propagation of culture strains, time and temperature of incubation of the culture, adjuvants and the methods of precipitation or adsorption on the adjuvant (Pittman, 1970). To produce better vaccines, Cameron (1976) stressed the need of controlling variations in different steps during production.

Chemical separation of the various cellular components has been the subject of many studies for more than 20 years (Munoz, 1963). The non cellular antigen prepared by the method of Fennel and Thielle (1951) was less reactive than the whole cell vaccine. The sub-cellular Pillemar antigen (Pillemar, Burell and Ross, 1947; Pillemar, Blum and Lepow, 1954) caused more reactions than the whole cell vaccine (Medical Research Council, 1959). The Pillemar antigen or stromata protective antigen (SPA) was prepared from extracts of sonicated B. pertussis cells adsorbed on human erythrocytes. The preparation was protective in the mouse potency assay and showed clinical efficacy in British trials (Medical Research Council, 1951, 1956). The extracts adsorbed to erythrocytes and sensitized mice against histamine showing that SPA was a mixture of cellular components including LPF.

Eli Lilly Company produced DPT adsorbed vaccine containing an acellular pertussis vaccine prepared from a trisodium phosphate extract of B. pertussis cells (Wiehl, Riley and Lapin.
Wyeth Laboratories, Inc. also prepared an experimental acellular pertussis vaccine which was potent in mouse potency assay and was antigenic in limited clinical studies but the reaction rates did not differ very much from whole cell vaccines (Brunnel, 1982).

Millman, Schuchardt and Gray (1962) developed a soluble pertussis vaccine by extracting cell envelopes of *B. pertussis* with sodium deoxycholate followed by adsorption on alum. At Behringwerke, a vaccine was prepared from urea salt extracts of *B. pertussis*. The vaccine was adsorbed on aluminium carrier and was potent in the mouse potency assay (Schwick, Helting and Zwisler. 1980).

Recently, an acellular pertussis vaccine has been developed in Japan (Sato et al. 1984). This vaccine contained two physico-chemically purified haemagglutinins of *B. pertussis* (FHA and LPF-HA). The component vaccine prepared from these two fractions was not only potent by the mouse protection test but also 10 times less toxic than the whole cell vaccine as judged by LPF, HSF and endotoxicity tests (Sato et al. 1984). The acellular vaccine gave less febrile reactions in children above 2 years of age and further studies are needed in the infants below 2 years to evaluate the reactogenicity of Japanese acellular vaccine (Noble. Bernier, Esber, Hardegree. Hinman. Klein and Saah. 1987; Cherry and Mortimer. 1987). This vaccine has been in use in Japan for immunization purposes since 1981 and recently efficacy of this vaccine has been shown in a number of preliminary trials in different countries (Isomura. Suzuki and Sato, 1985; Biritwum.

In addition to the development of acellular pertussis vaccine for the control of whooping cough, approaches to improve the existing whole cell vaccine are still in progress. Saran (1985) described acetone treated pertussis vaccine to be more potent and less reactogenic. These findings could not be confirmed in a later study (Gupta et al, 1987a). Glutaraldehyde and formaldehyde inactivated whole cell pertussis vaccines have
been found less toxic than the conventional heat inactivated pertussis vaccine (Gupta et al., 1987c,d). Iida and Horiuchi (1987) also found glutaraldehyde detoxified *B. pertussis* organisms used as vaccine less toxic than the formalin inactivated vaccine. The production of oral vaccines against pertussis have also been described which were found safe in a limited trial and produced local as well as serum immunity (Baumann, Binder, Falk, Huber, Kurz and Rosanelli, 1985; Vesselinova-Jenkins, 1985).

**Control testing of pertussis vaccine**

Potency and toxicity are the major factors of concern in the production of pertussis vaccine. A balance is kept between toxicity and potency because there is an upper limit set to the number of organisms present in a single dose of vaccine having minimum protective unitage. The various tests used widely for the control of pertussis vaccine have been described below.

**Opacity test**

As per the requirements laid down by W.H.O. (1979), the opacity of the final bulk in the volume recommended as a single human dose should not exceed 20 International Opacity Units (IOU) or 20X10⁹ organisms. The opacity is determined by visual comparison with the Fifth International Reference Preparation of Opacity (Perkins, Sheffield, Outschoorn and Hemsley, 1973). The United States Opacity Standard has also been used for opacity determination of bacterial suspensions by photometric methods (Pittman, Vorgas, Perez and Merino, 1974; Pittman, 1976; Seligman and Farber, 1976). The spectrophotometric methods have
been found convenient and accurate for opacity determinations of bacterial suspensions including pertussis vaccine (Gupta, Maheshwari, Bhandari, Sharma, Ahuja and Saxena, 1984; Gupta, Sharma, Ahuja and Saxena, 1986).

Potency test

In the Medical Research Council field trials, the protective efficacy of different vaccines was evaluated against a number of laboratory tests of which the mouse protection test was found to correlate best with the results of the field trials (Medical Research Council Report, 1959). Preston (1975) suggested that the mouse protection test should be supplemented by or even replaced by tests to check for an adequate content of the three agglutinogens 1, 2 and 3. The mouse protection test is influenced by a number of factors like mouse strain, challenge culture etc. (Pittman, 1967; Cameron, 1976). It is important to control each factor of the test and keep them constant between tests. Pittman (1967) suggested that the best strain of mouse was the one which was capable of being rendered highly sensitive to histamine by pertussis vaccine.

*B. pertussis* strain 18323 with agglutinogens 1, 2, 3, 4, 5 and 7 (Eldering, Holwerda and Baker, 1967) was introduced by Kendrick et al. (1947) in the collaborative study of 10 laboratories which established the feasibility of the intracerebral test. In a detailed study of the role of the challenge in the assay, Murata, Perkins, Pittman, Scheibel and Sladky (1971) found no indication that a uniform method for the preparation of the challenge reduced heterogeneity of results.
Other factors influencing the test were temperature of animal room, sex and randomization of mice in groups and in location of groups on shelves, medium for cultivation of culture, diluent for the challenge suspension, a fixed time of day for the challenge, discharge of each test by the same person from test to test (Pittman, 1951a; Pittman, 1970).

The intracerebral mouse protection test consists of intraperitoneal vaccination of the mice followed 2 weeks later by intracerebral challenge with live organisms. The mice are observed for mortality after 72 hours of the challenge for 11 days. The response of mice immunized with serial dilutions of vaccine, usually 4- or 5-fold (Wardlaw and Parton, 1983a) is compared with the corresponding dilutions of a standard vaccine to a certain level of challenge. A numerical value is assigned to the vaccine in terms of mouse protective unit as compared to the known unitage of the standard (Cameron, 1976). Despite variations in the potency test of pertussis vaccine (Finney, Holt and Sheffield, 1975; Cameron, 1979), this test has been found very useful in control of whole cell pertussis vaccine.

**Agglutination test**

Since the pioneer work of Evans and Perkins (1953;1954) on the agglutinin production in mice and the role of agglutinins in protection against whooping cough (Medical Research Council Report, 1959), the estimation of agglutinogen content of vaccines and evaluation of agglutinin response in animals as well as in man had great significance in the control of pertussis vaccines.

**Toxicity tests**

**Mouse Weight Gain test.** The toxicity testing of pertussis vaccine has always been a controversial issue. The mouse weight gain or mouse toxicity test is the only test approved by the W.H.O. (1979) and most of the National Control Authorities. This test is believed to reflect the toxicity of
pertussis vaccine (Pittman and Cox, 1965). The mouse weight gain test is influenced by a number of factors which include the mouse strain (Pittman, 1967; van Ramshorst, 1969; Pittman, 1980), size of cage, light and temperature of animal room (Cameron, 1969; Cameron, 1976; Cameron, Desormeaux, DeBellefeuille and Leclerc, 1984). The test requires that a group of 10 mice each weighing 14-16 g and injected intraperitoneally with half of a human dose of vaccine regains its original weight within three days of injection and achieves at least 60% of the weight of a control group of saline-injected mice seven days after injection (W.H.O., 1979) or gains an average of 3g per mouse seven days after injection (U.S. Requirements, 1983). van Ramshorst (1970) and Cameron (1977) recommended that a standard control vaccine should be included in this test instead of saline. The standard could not be used due to lack of agreement about what sort of vaccine should be used (Wardlaw and Parton, 1983a). Kurokawa (1984) stressed for the inclusion of reference vaccine in the toxicity tests and a toxicity reference vaccine is being used in Japan (Japanese Government Minimum Requirements for Biological Products, 1982). The injection of adsorbed vaccine caused greater initial weight loss as a result of the presence of adjuvant (Cameron, 1979).

Originally the mouse weight gain test was started to check the inactivation of heat labile toxin (Pittman, 1952; Pittman, 1970). The 24 hourly weight loss was mainly due to endotoxin (Ishida, 1968; Kurokawa, Ishida, Iwasa, Asakawa and Kuratsuka, 1968; Cameron, 1977). while slow weight gain and late (after 3 days) deaths could be due to LPF or pertussis toxin (Kurokawa et
al. 1968; Wardlaw and Parton, 1983a; Manclark and Cowell, 1984). There was no doubt that this test detected toxic lots of vaccine (Cohen, 1963). Cohen, van Ramshorst and Drion (1969) correlated depressed weight gain in mice with raised rectal temperatures and reactions in infants. Perkins, Sheffield, Miller and Skegg (1970) found that the weight gain test was able to distinguish the vaccines which gave more side reactions in children. Hilton and Burland (1970) confirmed the value and accuracy of the mouse weight gain test as an indicator of the reactivity of pertussis containing vaccines in children.

The other tests performed to investigate the toxicity of pertussis vaccine were HSF and LPF estimations. These tests have not been included in the W.H.O. requirements (W.H.O., 1979) but the tests have been used quite often for the toxicity testing of pertussis vaccine. These tests have been included in the Japanese Government Minimum Requirements for Pertussis Vaccine (1982) for the control of whole cell as well as acellular pertussis vaccines. Kurokawa (1984) has reviewed the toxicity testing of pertussis vaccine and emphasized the need of additional tests like HSF and LPF estimations to evaluate the toxicity of pertussis vaccines.

**Test for Histamine Sensitization.**

Sensitization of mice against histamine after intraperitoneal inoculation of vaccine could be detected between 24-48 hours that reached a peak on the 5th day, declined between 5-10 days and was not detectable after 15-30 days (Parfentjev and
Goodline, 1948; Pittman, 1951a; Parfentjev, 1955; Maitland et al., 1955; Pittman, 1957; Munoz, 1957; Kind, 1958; Munoz and Bergman, 1966). For the acellular pertussis vaccine, late HS (LHS) activity which reached a peak on 12th day of inoculation has also been described (Iwasa, Ishida, Asakawa and Akama, 1985). The LHS was more resistant to detoxification with formaldehyde than the 5 day early HS (EHS) activity. The LHS of whole cell pertussis vaccine was lower than the EHS while the reverse was the case for acellular vaccine (Iwasa et al., 1985).

Intravenous injection of soluble preparation of HSF developed sensitization within 90 minutes and it persisted for 84 days (Munoz and Bergman, 1966). Histamine sensitization has also been induced by the respiratory infection of mice with B. pertussis (Pittman, 1951a; Pittman, Furman and Wardlaw, 1980) or by intracerebral injection of vaccine (Munoz and Bergman, 1968). A number of factors influenced the result of HSF test (Kind, 1958; Preston, 1959). Strains of mice varied from high susceptibility to non-susceptibility (Pittman, 1967; Bergman and Munoz, 1968; Manclark, Hansen, Treadwell and Pittman, 1975). Female mice, 15-18 g, were more susceptible than male mice (Pittman, 1951a). Mice weighing 20 g or more have been found more susceptible than younger mice (Kind, 1958; Preston, 1959; Bergman and Munoz, 1964; Wardlaw and Parton, 1983b).

For the quantification of histamine sensitization content in the vaccine, Parfentjev and Goodline (1948) employed pertussis vaccine at constant dose and varied the dose of histamine challenge. This method was not found satisfactory
because at times higher histamine challenge dose caused fewer deaths than the lower challenge dose (Pittman, 1951a; Preston, 1959; Munoz and Hestekin, 1966). Pittman (1975) described the method for determination of HSF in which 5-fold dilutions of vaccines were inoculated. The mice were challenged 5 days after inoculation and 50% histamine sensitizing dose (HSD50) was calculated (Gupta et al., 1986; 1987c). Ishida, Kurokawa, Asakawa and Iwasa (1979) described a modified HSF estimation test in which change in rectal temperature of inoculated mice was recorded after histamine challenge.

Several workers found parallelism between HSF and protective activity (Pittman, 1951b; Maitland et al., 1955; Frappier and Guérault, 1955; Maitland and Guérault, 1958; Barta, 1963; Munoz and Hestekin, 1963; Pittman, 1975; Gupta, Sharma, Ahuja and Saxena, 1985), while others reported that HSF and protective activity were independent of each other (Fishel, 1956; Dolby, 1958; Sutherland, 1963; Nagel, 1967; Sato and Nakase, 1967; Saran, 1982). Now it is clear that histamine sensitization of mice and protection of immunized mice against B. pertussis organisms are due to pertussis toxin (Wardlaw and Parton, 1983b; Manclark and Cowell, 1984; Pittman, 1984b; Sato and Sato, 1985; Manclark and Burns, 1985; Robinson et al., 1985; Munoz, 1985).

Although the relation of HSF to human reactions has not been clearly defined, results of Cohen (1968) showed that acid precipitated bacteria used in the vaccine having higher HSF content than those of centrifuged sedimented bacterial vaccines
were more toxic for mice. Perkins et al (1970) found histamine sensitizing test comparable with the mouse weight gain test for distinguishing the test vaccines giving higher reactions in children. The relation of histamine sensitizing unit and protective unit has also been established by many workers (Pittman, 1951b; van Hemert, van Wezel and Cohen, 1964; Pittman, 1975). The HS content of pertussis vaccine had great significance as Sanyal (1960) found that histamine sensitization in children was increased after pertussis infection.

Test for LPF. One of the characteristic biological activity of pertussis toxin has been the marked leucocytosis which was observed in a number of species including lampreys, pigs, calves, sheep, rabbits, guinea pigs, rats, mice, monkey and man (Olson, 1975). Out of these, the mouse has been used most widely for detecting LPF activity of pertussis vaccines or pertussis toxin. The marked influences of age and mouse strain observed with HSF estimation did not affect the LPF response (Wardlaw and Parton, 1983b).

After intravenous injection, the leucocytosis occurred most rapidly (between 2nd and 5th day) while intraperitoneal injection produced less substantial response and subcutaneous route was ineffective (Wardlaw and Parton, 1983b). Pittman et al (1980) observed leucocytosis after sublethal pulmonary infection of mice which lasted for 5 weeks. During leucocytosis response in mice, about 60-70% of the cells were small lymphocytes (both T cells and B cells) and the relative increase
in B lymphocytes, was greater (Morse and Morse, 1976).

The assay of LPF activity of pertussis vaccine or cell extract is complicated by endotoxin which also affected the white cell count in the blood (Wardlaw and Parton, 1983b). The maximum leucocytosis occurred 24 hours after intraperitoneal injection of purified endotoxin whereas the leucocytosis produced by endotoxin free LPF reached a peak at 3-4 days after injection (Kurokawa, Ishida, Asakawa and Iwasa, 1978). Thus by making the total leucocytes counts on day 3 or 4 post injection, the interference by endotoxin in LPF assay was minimized (Kurokawa et al, 1978).

Control of Acellular Pertussis Vaccine

The various tests for quality control of whole cell pertussis vaccine particularly the standard intracerebral mouse protection test and the active mouse weight gain test have not been appropriate for the control of acellular pertussis vaccine (Manclark and Cowell, 1984; Memorandum W.H.O., 1985). The control testing of the acellular vaccines would involve specific physical, chemical, biological and immunological assays to characterize the individual components of acellular vaccine (Manclark, 1985).

The various components including FHA and pertussis toxin or LPF could be detected by polyacrylamide gel electrophoresis (PAGE), high pressure liquid chromatography (HPLC) and simple haemagglutination assay (Cowell et al, 1982; Robinson and Hawkins, 1983; Manclark, 1985). The enzyme linked immunosorbent assay (ELISA) has also been developed for the assay of FHA and
pertussis toxin (Irons and MacLennan, 1979a; Askelöf et al., 1982; Sato et al., 1983; Sekura et al., 1983; Burstyn, Baraff, Peppler, Leake, Geme and Manclark, 1983). The specific biological activities of pertussis toxin like leucocytosis, histamine sensitization, action on Chinese hamster ovary (CHO) cells etc. may also be utilized for its assay before detoxification. After detoxification, these assays would help in determining the residual toxicity and reversal of toxicity (Manclark, 1985). The toxicity due to dermonecrotic toxin may be detected in the suckling mice and that due to endotoxin could be detected by measuring the pyrogen content in rabbits or by Limulus Amoebocyte Lysate (LAL) test (Japanese Government Minimum Requirements for Biological Products, 1982; Memorandum W.H.O., 1985).

To check the immunogenicity of various components, serum of immunized animals would be assayed for anti-pertussis toxin and anti-FHA antibodies by neutralization test in CHO cells and ELISA (Gillenius et al., 1985; Tiru, Jäätmaa, Gillenius and Askelöf, 1985; Vernon, Wiener, Urbano and Levner, 1986). Intranasal or aerosol challenge methods might also be useful for determining the immunizing potency of acellular preparations (Sato, Izumiya, Sato, Cowell and Manclark, 1980).

Effects of inactivating agents and preservatives on the quality of pertussis vaccine

Inactivating Agents

Inactivation of B. pertussis organisms or detoxification
of pertussis toxin is an important step in the production of whole cell or acellular pertussis vaccine. Various agents used for inactivation/detoxification affect the potency and toxicity of the pertussis vaccine.

The inactivation of the organisms in the manufacture of pertussis vaccine has generally been carried out by heating at 56°C for 30-60 minutes (Pittman, 1952; Cohen, 1963), for 20 minutes (Gardner, 1967) and for 10 minutes (van Hemert, 1971) or at 34°C for 24 hours (Pittman, 1952). Pittman (1970) reported that heating at 56°C was the most effective method for detoxification and heating at 37°C was not suitable because of the low stability. van Hemert (1971) found that heating at 56°C beyond 10 minutes gave no appreciable decrease of toxicity. Joó (1970) found that repeated heating decreased toxicity but affected potency also. Ultraviolet irradiation was unsatisfactory for detoxification or preservation of potency (Pittman, 1952).

Formalin when used for detoxification reduced toxicity but caused loss of potency also (Relyveld, 1970). Loss of potency of formol treated vaccine in comparison with heated vaccine has also been observed by Yoshioka, Takatsu, Kawahira and Takahashi (1967). Kendrick, Eldering, Hornbeck and Baker (1955) and Gupta et al. (1987a) noted no deleterious effect of formalin on potency while other workers reported the deleterious effect of formalin on antigenicity (Billaudelle, 1961; Joó. Pusztai and Junász, 1961; Munoz and Hestekin, 1966). However, formalin treatment for 24 hours has been used by certain manufacturers for the production of pertussis vaccine (Zakharova.
1958; Muggleton, 1967). Histamine sensitizing activity decreased by the action of formaldehyde at 37°C at a faster rate than the protective activity (Munoz and Hestekin, 1966; Gupta et al., 1987c). Recently Cameron (1983) and Gupta et al. (1987a) have found formalin as a better inactivating agent for the manufacture of pertussis vaccine. It has been reported that formalin detoxified vaccine was less toxic than the conventional heat inactivated vaccine in the mouse weight gain toxicity test while the potency and stability of formaldehyde inactivated pertussis vaccine were similar to those of heat inactivated pertussis vaccine (Gupta et al. 1986; 1987a). Sato et al. (1984) prepared the component pertussis vaccine using formalin as detoxifying agent.

Glutaraldehyde has been found as a better detoxifying agent for the manufacture of diphtheria and tetanus toxoids (Relyveld, 1975; Relyveld, 1978; Relyveld, 1980; Relyveld and Ben-Efraim, 1983), but it has not been used much for the preparation of pertussis vaccine. Relyveld, Girard, Cheyroux, Asso and de Rudder (1974) reported that 0.0131M glutaraldehyde killed B. pertussis strain 134 organisms at a concentration of 200 opacity units in one minute at 37°C and the potency of this preparation was about three times less than that of the heat inactivated (56°C for 30 minutes) vaccine. The potency of B. pertussis strain 509 organisms at a concentration of 84 opacity units detoxified with 0.00131M glutaraldehyde at 37°C for one minute was equal to that of the heat inactivated preparation (Relyveld, Girard and Desormeau-Bedot, 1973). Relyveld and Ben-Efraim (1983) stated that the optimal conditions for preparation of B. pertussis
vaccine with glutaraldehyde have not yet been fully determined. Recently glutaraldehyde inactivated pertussis vaccine has been found a safe, potent and stable vaccine when compared with the heat or formalin inactivated pertussis vaccine (Iida and Horiuchi, 1987; Gupta et al., 1987a,c,d). Glutaraldehyde has also been used for toxoiding pertussis toxin or pertussigen (Munoz, Arai and Cole, 1981; Munoz, Arai, Bergman and Sadowski, 1981; Cowell et al., 1982; Munoz and Arai, 1982; Robinson and Hawkins, 1983; Roumiantzeff et al., 1986).

The thiomersal may also be used for inactivation of B. pertussis for the production of vaccine. The original method of detoxification was with 0.02% merthiolate at 4°C for several months (Pittman, 1970). Thiomersal had minimum effects on the toxicity and potency of the vaccine (Gupta et al., 1987a,c). Gardner (1967) showed that thiomersal in concentration of 1:1000, 1:5000 or 1:10,000 did not reduce the potency of pertussis vaccine. Munoz and Hestekin (1966) also found that HSF and protective activities were not demonstrably affected by 1:10,000 concentration of merthiolate. It was presumed that merthiolate inhibited catalase activity and prevented bacterial lysis (Pontecorvo, 1967).

Acetone has been used for the preparation of killed typhoid vaccine (W.H.O., 1967). By acetone treatment of B. pertussis, Munoz and Hestekin (1963) prepared extracts having high histamine sensitizing and protective activities. Saran (1985) prepared an acetone treated pertussis vaccine with high potency and low toxicity as judged by mouse weight gain test and histamine
sensitizing activity. These findings could not be confirmed in a subsequent study (Gupta et al., 1987a).

Preservatives

For several years thiomersal has been used as a preservative in pertussis vaccine. Pittman (1952) found that the vaccines preserved with either formalin or phenol were less stable with regard to potency than with thiomersal. Kendrick et al. (1955) reported that the merthiolate-preserved vaccines were stable in potency with or without heating at 56°C. Vaccine containing merthiolate was stable in comparison to vaccine without a preservative (Gardner and Pittman, 1965). Merthiolate is used in DPT vaccines as preservative but not in poliomyelitis vaccine because free mercury ions are deleterious on its potency. Benzethonium chloride, an acceptable preservative in poliomyelitis vaccine, has been used in DPT-polio (quadruple) vaccines. Studies on a number of lots of quadruple vaccine prepared in the U.S.A. disclosed that the pertussis component was unstable in the presence of benzethonium chloride and the rate of loss in potency was about 6% per month (Edsall, McComb, Wetterlow and Ipsen, 1962; Pittman, 1962). Benzethonium chloride and parabens have been found to be deleterious to the potency of pertussis vaccine (Olson, Eldering and Graham, 1964; Gardner and Pittman, 1965). Addition of cations prior to treatment with benzethonium chloride inhibited deleterious action (Olson et al., 1964). Vaccines prepared by formol treatment and preserved with benzethonium chloride did not show a higher loss of potency than when stored without preservative at 35°C (Pontecorvo, 1967). Relyveld (1970)
stressed that the method of preparation of vaccine has been an important point in the action of benzethonium chloride.

Merthiolate has presently been accepted as the best preservative agent for pertussis vaccine. Merthiolate had one more advantage that severe shock in mice induced by formalin-treated vaccine was prevented by the addition of merthiolate (Muggleton, 1967).

**Effect of adjuvants**

Usually an adjuvant is introduced into a vaccine to enhance the immune response of the host, to localize injected material and to prevent severe systemic reactions otherwise observed with non-adjuvanted (non-adsorbed) vaccines. Aluminium hydroxide or phosphate have mostly been used as adjuvants in the DPT vaccine (Wardlaw and Parton, 1983a). Calcium phosphate has been found as a better adjuvant for DPT vaccine by Relyveld and Raynaud (1967). Pertussis bacteria got adsorbed onto the calcium phosphate adjuvant.

Pertussis vaccines adsorbed or precipitated with aluminium salt compounds provided better and longer protection (Sako, Treuting, Witt and Nichamin, 1945; Sako, 1947) and were less reactive (Burland, Sutcliffe, Voyce, Hilton and Muggleton, 1968; Hilton and Burland, 1970) than plain saline suspended preparations. However, as claimed by Aprile and Wardlaw (1966) that it remained to be proved, whether or not the aluminium containing adjuvants were able to increase potency for man.

There are a number of conflicting reports about the use of adjuvants in DPT vaccines. Pittman (1954) found that vaccines
had 1.7 times more potency for the mouse after adsorption than before adsorption whereas Cameron (1976) found that the adjuvant had almost negligible effect on the immune response. The adjuvant aluminium hydroxide actually depressed the protective response in mice (Cameron and Knight, 1972; Novotny and Brookes, 1975). Therefore, it would require a number of assays to validate the claims for adjuvant increasing the potency. The adjuvant had no effect on the potency of pertussis vaccine was supported by the fact that adsorbed and non-adsorbed vaccines are regularly included in the same assay and most of the reference preparations are non-adsorbed whereas the majority of vaccines are adsorbed (Cameron, 1979). If there had been an enhanced response with the adsorbed vaccines, the slopes of the dose response curves could never show parallelism as in the case of diphtheria and tetanus toxoids where plain and adsorbed toxoids could never be compared in the same assay. van Ramshorst (1970) found no difference in 'b' values of plain pertussis vaccine and DPT vaccines adsorbed on aluminium phosphate suggesting that from the point of view of parallelism a plain reference vaccine could be used for estimating the potency of vaccines containing aluminium phosphate and other antigenic components. It was reported, further, that there was no difference between the potencies of pertussis components present in aluminium phosphate adsorbed DPT polio vaccines and plain pertussis vaccines. However, in a recent study the intracerebral potency of different types of pertussis vaccines was found enhanced by the aluminium phosphate adjuvant (Gupta et al. 1987b).
The aluminium adjuvants apparently did not affect the HSF activity of pertussis vaccine (Pittman, 1951b; Cohen, 1963). Preston (1979) advocated that the need to incorporate the adjuvant in pertussis vaccine is beyond question. He concluded that the argument that aluminium adjuvant did not increase and might even decrease the potency of pertussis vaccine in mice was merely one further reason for abandoning the mouse protective test because the children were not going to be vaccinated intraperitoneally. Aluminium hydroxide adsorbed vaccines have been found better by Preston, Mackay, Bamford, Crofts and Burland (1974) and Preston (1976) with respect to higher agglutinin production in children as compared with the non-adsorbed vaccines.