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ISOLATION AND CHARACTERIZATION OF SUBCELLULAR (PEAK "K") COMPONENTS OBTAINED DURING THE PURIFICATION OF PERTUSSIS TOXIN

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ISOLATION AND CHARACTERIZATION OF SUBCELLULAR (PEAK "K") COMPONENTS OBTAINED DURING THE PURIFICATION OF PERTUSSIS TOXIN

By

Elizabeth Ann Stephens

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF SUBCELLULAR (PEAK "K") COMPONENTS OBTAINED DURING THE PURIFICATION OF PERTUSSIS TOXIN

By

Elizabeth Ann Stephens

This work was designed to determine whether an eluate from pertussis toxin purification, "Peak K", contained protective proteins or other components that would improve the efficacy of an acellular pertussis vaccine.

"Peak K" eluate was separated by gel filtration with Sephacryl S-300 into subfractions Ks-1 and Ks-2. Preliminary work indicated that these two fractions contained some component(s) that increased the protective activity of pertussis toxin. Further subdivision was accomplished by Reverse Phase-High Performance Liquid Chromatography and discontinuous polyacrylamide gel electrophoresis.

RP-HPLC fractions were selected for in vivo studies in animals to determine their activity. The results indicated fractions that appeared to express protection by the mouse protection assay and enhanced antibody induction to rabies vaccine by the Rapid Fluorescent Focus Inhibition Test (Rffit).

Further purification resulted in reduction of protective/immunoenhancing activity that had been seen previously. The contributing factor responsible for the observed activity seemed to be associated with the presence of endotoxin. Selected RP-HPLC fractions appeared to contain molecular weight peptides reported by others to be protective or virulence factors; however, their separation from endotoxin, pertussis toxin and filamentous hemagglutinin factor (FHA) rendered them less active in animal tests.

Studies designed to characterize the cellular immune response elicited by various peptides did not produce the expected results. Rabbit peripheral blood lymphocytes failed to respond to blast transformation under the conditions of these experiments. The CHO cell and ELISA data indicated humoral response to experimental component vaccines.

Identification and characterization of some peptides was done by Western blot and immunostaining with monoclonal antibodies. A 69-kDa protein described by others to be associated with protection was identified and shown to have some similar characteristics. A 30-kDa peptide reported to have adjuvant activity was tentatively identified. Additional peptides at 216-kDa and 44-kDa have been reported to be associated with adenylate cyclase toxin; however, that relationship was not confirmed by this research.

To my husband Sam

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INTRODUCTION

Whooping cough is a contagious disease caused by Bordetella pertussis, a gram negative organism characterized by its affinity for mucous membranes of the respiratory epithelium. Humans appear to be the only natural host, in whom a characteristic set of symptoms is displayed, especially in unimmunized infants and young children. The disease is still considered to be a major cause of morbidity and mortality in this age group throughout the world, especially in regions with inadequate immunization programs. The World Health Organization estimates 600,000 deaths yearly due to pertussis, virtually all deaths occurring in unimmunized infants (Six Killers of Children, 1987).

During the early 1970's the success rate of the whole cell pertussis vaccine was shrouded in controversy relating to vaccine effectiveness versus adverse events from immunization. Widely publicized, but rare neurological complications presumably thought to be due to the toxic nature of the whole cell vaccine led to reduced acceptance of the vaccine and ultimately the reappearance of epidemics. This controversy over vaccine preventable diseases and the adverse events due to immunization has resulted in renewed

interest in safer vaccines (Report of the Task Force on Pertussis and Pertussis Immunization, 1988).

Bordetella pertussis contains many antigenic and biologically active factors, the effects of which have only been demonstrated in animal models. The mouse intra-cranial (IC) challenge protection test has been used to evaluate pertussis vaccine potency (Kendrick et al., 1947), but it does not reflect the course of disease in man. There is increasing evidence of its inadequacy to demonstrate protection of mice against IC challenge with new acellular vaccines (World Health Organization, 1979). The new acellular vaccines have been aimed at the characterization and purification of selected protein components and the reduced influence of the very toxic lipopolysaccharide-endotoxin of the organism. An acellular vaccine developed in Japan contains filamentous hemagglutinin (FHA), formalin detoxified pertussis toxin (PTx) and other proteins obtained from the supernatant of the organism, combined with diphtheria and tetanus toxoids adsorbed to aluminum hydroxide/aluminum phosphate (Sato et al., 1984).

Currently a component, acellular, vaccine is being developed by the Michigan Department of Public Health. The purpose of this research in conjunction with that end was to locate and characterize any components associated with one of the fractions, "Peak K", obtained during the purification of pertussis toxin. Early work indicated that some compo-

nent(s) in peak "K" appeared to increase the protective or immunoenhancing ability of the purified pertussis toxoid.

The research presented here identified certain components in peak "K" that appeared to be associated with protective or immunoenhancing activity. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) was used to analyze and further subdivide fractions obtained from a sephacryl S-300 gel filtration column that were designated Ks-1 and Ks-2. The RP-HPLC fractions were analyzed by discontinuous polyacrylamide gel electrophoresis (SDS-PAGE) to locate areas of protein concentration. In vivo studies in mice, guinea pigs and rabbits were done on selected fractions to determine their associated biological activity.

The Western blot and immunostaining techniques were used with various monoclonal antibodies to determine whether the strain being used for the development of the MDPH acellular vaccine contained peptides that had been reported on by others to have protective or virulence characteristics. Certain peptides identified by the technique were isolated and assayed for biological activity by incorporation into experimental acellular vaccines for protective activity.

LITERATURE REVIEW

Whooping cough (pertussis) is a contagious disease of the tracheobronchial tree caused by a gram-negative bacillus, Bordetella pertussis (Bordet and Gengou, 1906). The organism is characterized by an affinity for mucous membranes of the respiratory epithelium in humans, in whom a characteristic set of symptoms is displayed especially in unimmunized infants and young children.

Whooping cough has been, and still is a major cause of morbidity and mortality in young children, especially in countries where there is inadequate immunization programs. The World Health Organization estimates 600,000 deaths yearly due to pertussis, virtually all occurring in unimmunized infants (Six Killers of Children, 1987). The first written description of pertussis appeared in 1578, when an epidemic occurred in Paris (Cone, 1970). The causative agent was isolated by Bordet and Gengou (1906) from the sputum of children with classic whooping cough. Bordet and Gengou also demonstrated that suspensions of bacterial cells grown on a medium consisting

of starch infusion, glycerol and defibrinated blood (B-G agar) fixed complement in serum from convalescent children (Manclark and Cowell, 1984).

Current taxonomy specifies three species in the genus Bordetella of which B. pertussis is the etiologic agent of human pertussis; B. parapertussis is responsible for mild pertussis-like diseases in humans; and B. bronchiseptica is an animal pathogen that causes such diseases as kennel cough in dogs, snuffles in rabbits and atrophic rhinitis in swine (Manclark and Cowell, 1984; Mooi, 1988). A fourth species B. avium, which causes respiratory disease in birds, has also been mentioned (Kersters et al., 1984; Mooi, 1988). The four species demonstrate different phenotypes; however, DNA homology studies suggest that B. pertussis, B. parapertussis and B. bronchiseptica express genotypic similarities (Kloos et al., 1981).

Clinically the disease is characterized by an incubation period of approximately 10 days followed by a catarrhal stage which lasts 1-2 weeks and then by the paroxysmal stage of 1-6 weeks with the violent, convulsive cough and vomiting episodes. The patients tongue is fully protruded, fluids stream from the eyes, nose and mouth, and the face appears cyanotic. The "whooping" sound is produced as a result of inspired air rushing past a narrowed glottis. The convalescent period of 1-6 weeks follows before the individual returns to normal. Since a variety of viruses, e.g., adenovirus, can cause similar coughing episodes, it is recommend-

ed that a positive bacterial culture along with the duration of the paroxysmal stage of 3 weeks or greater be used as a positive diagnosis (Preston, 1988).

In the United States where mass immunization has successfully controlled pertussis, the number of cases went from 115,000-270,000, including 5000-10,000 deaths per year, to 1200-4000 cases with 5-10 deaths per year (Centers for Disease Control, 1982,1984, 1987a,b). However, since the early 1970's the success rate has been shrouded in controversy relating to vaccine effectiveness versus adverse events from immunization. Widely publicized but rare neurological complications, presumably due to the toxic nature of the whole cell vaccine, e.g., 1 in 110,000 for serious neurological disorders and 1 in 310,000 for persistent damage, has led to reduced acceptance of the vaccine in such industrialized countries as the United States, the United Kingdom, Sweden and Japan and ultimately to the reappearance of epidemics (Alderslade et al., 1981). The controversy over vaccine preventable diseases and the adverse events of some vaccines has resulted in a renewed interest in the development of vaccines which appear to be associated with fewer adverse events.

ETIOLOGY

Several types of adenoviruses (Baraff et al.,1978), B. parapertussis and B. bronchiseptica (Report of the Task Force on Pertussis and Pertussis Immunization, 1988) have been implicated in pertussis-like illnesses. The majority of cases and all major epidemics have been caused by Bordetella pertussis (Mooi, 1988). Small gram-negative coccobacilli (0.2 to 0.8 um) were demonstrated in smears of nasopharyngeal swabs, in sputum of patients with clinical pertussis, and in stained lung sections from children who had died of pertussis (Manclark and Cowell, 1984).

The growth requirements of the organism are not completely understood; however, it appears that the genus Bordetella requires niacin or nicotinamide. Carbohydrates, lactate, pyruvate, acetate and intermediates of the Embden-Meyerhoff pathway do not appear to be required (Manclark and Cowell, 1984). A modification to the Stainer and Scholte (1970) growth medium containing nicotinamide, glutathione, ascorbic acid, a sulfhydryl containing amino acid (e.g, cystine, cysteine), glutamic acid, and/or proline in a sodium glycerophosphate buffer rather than Tris buffer has been described by Lothe et al.,(1985). B. pertussis grows aerobically on Bordet-Gengou agar at 35.5^{o}C and produces a punctiform, convex, glistening, translucent colony with an entire margin and hazy zone of hemolysis (Manclark and Cowell, 1984). Leslie and Gardner (1931) described four

growth phases, I, II, III & IV, which the organism displays in vitro. Changes from phase I to IV reflect a loss of virulence. Cells in phase I express a number of biologically active components or virulence factors that may have important roles in the host-parasite relationship.

EPIDEMIOLOGY

Man appears to be the only natural host for Bordetella pertussis in whom it causes a typical clinical syndrome followed by a high degree of immunity. The epidemiology is determined by factors that influence transmission of the disease within the community, e.g., infectivity of the organism, number of contacts and degree of exposure to infected individuals, and the level of immunity to pertussis in exposed contacts. The proportion of a population susceptible at a given time depends on birthrate, administration of vaccine, efficacy of the vaccine, incidence of disease, rate of decline in immunity following disease or vaccination, and the frequency of unsuspected infection boosting immunity (Thomas, 1989).

Transmission from an infected individual to a susceptible host presumably occurs by direct inhalation of infectious airborne respiratory secretions, or from the infected secretions contaminating the environment and the host being indirectly infected by the hands. The time between acquisition of infection and subsequent transmission to another susceptible individual varies between 1 and 4 weeks (Gordon

and Hood, 1951), with symptoms appearing approximately 7-14 days post infection (Stocks, 1933). Recovery of B. pertussis from infected individuals is highest during the first week of illness, but rapidly declines during the second and third weeks (Gordon and Hood, 1951; Lawson, 1933; Kristensen, 1933). Diagnosis of classical whooping cough is therefore made on the clinical appearance of the patient in the paroxysmal stage and bacteriological isolation from nasopharyngeal exudate.

While both natural disease and vaccination confer immunity, the natural disease confers almost absolute protection during childhood with protection from vaccination being less complete. The immunity from vaccination and natural infection tends to decline over time (Lambert, 1965; Jenkinson, 1988). When older children or adults with declining immunity are exposed to pertussis, they may develop typical pertussis or an atypical illness of shorter duration. The atypical illness and asymptomatic infection most likely occurs frequently and may be responsible for boosting immunity as shown by the presence of IgG and IgA antibodies to B. pertussis in the sera of individuals with no recognized symptoms (Nagel and Poot-Scholtens, 1983; Prasad and Saran, 1983).

PATHOLOGY AND PATHOGENESIS

Bordetella pertussis is a noninvasive organism that attaches to respiratory epithelial cells and exerts its

effects from the surface of the cells through the action of several bacterial toxins (Linneman, 1979). The organism expresses marked tropism for the ciliated columnar epithelial cells of the trachea and bronchi (Linneman, 1979; Tuomanen and Weiss, 1985; Weiss and Hewlett, 1986) where they attach and multiply on, between and at the base of cilia causing ciliostasis, cell death and shedding. The appearance is that of a necrotizing inflammation of the air passages of the nose and throat with copious amounts of exudation containing mucous and epithelial cells (Collier et al., 1977; Muse et al., 1977; Matsuyama, 1977). Due to the occlusion of the bronchi by the mucous, atelectasis and pneumonia may occur. The occurrence of a significant rise in body temperature suggests infection with a second organism. Reports of a rare but permanent central nervous system involvement, especially in infants, generally occurs during the paroxysmal stage in the form of convulsions and coma (Centers for Disease Control, 1984; 1987). Other neurologic complications such as seizures, partial paralysis, ataxia, aphasia, blindness and deafness have been reported. Prolonged and violent expiratory efforts during the coughing spasms may have secondary effects in the form of epistaxis, melena, petechia, subdural hematoma and spinal epidural hematoma. The resulting pressure has been shown to cause umbilical or inguinal hernias, rectal prolapse, pneumothorax and emphysema (Report of the Task Force on Pertussis and Pertussis Immunization, 1988). The pathologic findings

associated with pertussis have not been inflammatory in nature (Miller et al., 1956). Gross examination of the brain in some cases revealed edema in the meninges and occasional brain hemorrhages in the cerebral hemispheres that were vascular and degenerative. These changes were more consistent with anoxic brain damage (Dolgopol, 1941). At present the cause of pertussis encephalopathy is not definitive, but is most likely due to anoxia associated with coughing paroxysms. Other systemic effects of pertussis are not as clearly defined. Weight loss and hypoglycemia may be due to the poor nutritional status rather than a systemic toxicity. The increase in circulating peripheral lymphocytes or lymphocytosis is presumably due to the effects of the pertussis toxin (Report of the Task Force on Pertussis and Pertussis Immunization, 1988).

Bordetella pertussis contains many antigenic and biologically active factors, the effects of which have only been demonstrated in animal models. It must be kept in mind that since man is the only natural host of B. pertussis, studies done in animals may not be completely relevant to the disease process in man. Colonization of the respiratory tract with B. pertussis has been done in many animals from primates to rodents. The characteristic coughing and vomiting have not been reported in animals; however, infection in the marmoset (Stanbridge and Preston, 1974; Preston and Stanbridge, 1976;) and the rabbit

(Preston et al., 1980) has shown some similarities to man, e.g., catarrh, persistence of colonization in the nasopharynx for many weeks, change of serotype during colonization, and inability of the degraded type 1 organism to establish itself as the predominant serotype. All three hosts produce a similar range of agglutinin response to vaccination; and active immunization shows evidence of serotype specificity.

The mouse intra-cerebral (IC) challenge protection test has been used for many years to evaluate pertussis vaccine potency (Kendrick et al., 1947), and has an established correlation between the level of protection in mice with that in human infants; however, it does not reflect the course of disease in man. There is evidence of the inadequacy of the mouse (IC) potency test to demonstrate protection with new acellular vaccine preparations (World Health Organization, 1979). There is also evidence that potency of component vaccines, as determined by the mouse potency test, is comparable to existing whole-cell vaccines (Manclark and Cowell, 1984). The pulmonary infection model in mice (Pittman et al.,1980; Sato et al.,1980; Robinson et al., 1985; Cameron, 1988) has gained renewed interest for evaluating newer vaccines; however, neither the World Health Organization nor the Bureau of Biologics in the USA has established recommended standards or test methods for potency and toxicity of acellular vaccines. Before any testing protocol for potency and safety of acellular

vaccines can be adopted, it is essential to establish correlation with performance in infants (Cameron, 1988). The pulmonary model differs from the natural human infection (Carter and Preston, 1981) in that organisms of serotype 1 can infect and even kill mice, and the tendency to change serotype during colonization appears to be less. There is also a limited range of agglutinin response to vaccination (Agarwal and Preston, 1976). Andersen and Bentzon (1958) demonstrated that serotype specificity of active immunity was not seen in the mouse. It appears that there are marked differences between mouse and man in the response to both pertussis vaccine and to infection. There is evidence that these hosts differ in their reactions to some of the toxic products of B. pertussis; and, therefore, experimental evidence on virulence factors should be interpreted with caution.

ANTIGENIC AND BIOLOGICALLY ACTIVE FACTORS

Colonization factors. The first step in the disease process is adherence to host epithelial cells. Several classes of protein are presumably involved in adherence of B. pertussis to host ciliated epithelium: the filamentous hemagglutinin (FHA); pertussis toxin(s); fimbriae and recently a 69-kDa outer membrane protein may also be involved in adherence.

Filamentous hemagglutinin: Sato et al., (1983) described FHA as being a hydrophobic, rod-like molecule (2 x

100nm) which agglutinated a variety of erythrocytes. The FHA molecule is a heterogeneous collection of polypeptide species with a primary polypeptide at 220-kDa, and degradation products at 140, 125, and 98-kDa on SDS-PAGE (Irons et al., 1983). Tuomanen and Hendley (1983), demonstrated by transmission electron microscopy and by analysis of transposon mutants (Tuomanen et al., 1985) that the specific adherence of B. pertussis to ciliary tufts on segments of human bronchial epithelium was mediated by FHA. Using WiDr cells, an epithelium like cell line from a human intestinal carcinoma, and ^{35}S-methionine labeled B. pertussis, Urisu et al., (1986) demonstrated that FHA deficient mutants did not adhere as well as the parent strain. Goat antibody to FHA inhibited adherence of Tohama I strain in a dose related manner, but not the FHA deficient mutant Tohama 325. Normal goat antiserum, goat anti-pertussis toxin and goat anti-serotype 2 fimbriae had no effect on adherence. Mice immunized either IP or IM with FHA prior to aerosol challenge with B. pertussis Tohama I strain, had significantly reduced bacterial colonization in the lungs (Kimura et al., 1990).

Pertussis toxin (PT): Pertussis toxin, also called histamine-sensitizing factor (HSF), lymphocyte promoting factor (LPF), islet-activating protein (IAP) or pertussigen, has also been implicated in adherence. PT consists of 6-8nm spheres of 107,000 MW (Sato et al., 1983) or 117,000 MW composed of 5 subunits: S1 (28-kDa), S2 (23-kDa), S3 (22-

kDa), S4 (11.7-kDa) and S5 (9.5-kDa) (Tamura et al., 1982). Mutants deficient in pertussis toxin were unable to bind to ciliary cells (Tuomanen et al., 1985). Tuomanen and Weiss (1985) demonstrated that: (a) exogenous PT or FHA, when incubated with deficient mutants, was able to reconstitute the ability of mutants to adhere, (b) the presence of both FHA and PT seem to be required for effective adherence by acting as bridges between two adhering cells, (c) polyclonal antibody to FHA and PT blocked adherence.

Fimbriae: There appears to be some controversy and confusion over the nature of fimbriae and agglutinogens and their association with surface structures. Agglutinogens are both fimbrial and bacterial cell surface associated components, detected by direct agglutination of the organism with type-specific antisera. Pertussis expresses several agglutinogen types; however, three major agglutinogens (1,2 & 3) occur in various combinations in the majority of strains. Fimbriae belong to a class of extracellular pili proteins that may or may not be involved in the attachment of B. pertussis to host tissues. According to Ashworth et al., (1982, 1985) there are two antigenically distinct fimbriae produced by B. pertussis which were first identified as agglutinogens. Ashworth et al. (1985) claimed that the two fimbriae corresponded to agglutinogens 2 and 3, while Cowell et al. (1987) claimed they corresponded to agglutinogens 2 and 6. At least three distinct fimbrial subunit genes were detected in B. pertussis strains (Mooi et

al. 1987), such that a single strain of B. pertussis could produce one fimbrial serotype, both serotypes 2 and 3 or no fimbriae at all. According to Frederikson et al. (1985) agglutinogens are proteins with MW between 10 and 23 -kDa that generate agglutinating antibodies that are type specific, i.e, provide the serological markers (Andersen, 1953; Eldering et al. 1957) that may be important protective antigens. Preston et al. (1990) located three agglutinogens by immunogold labelling and electron microscopy with three serotypes of Bordetella pertussis (types 1,2,3; 1,2 and 1,3). Agglutinogen 1 was found on fimbriae and the cell surface of types 1,2,3 and 1,2; but, on the cell surface only of non-fimbriate type 1,3 organisms. Agglutinogen 2 was found only on fimbriae. Agglutinogen 3 was found on the cell surface only, even of fimbriate bacteria type 1,2,3. A role for fimbriae in mucosal attachment is a source of controversy. Urisu et al. (1986) showed that the Fab fragments of goat antibody to serotype 2 fimbriae did not block adherence. Also, an FHA positive strain lacking fimbriae showed high adherence, while a fimbriated FHA-deficient mutant adhered poorly to the WiDr cell line. Because fimbriae from many bacterial pathogens have been shown to mediate adherence (Mooi and de Graaf, 1985), it has been assumed that B. pertussis fimbriae might have a similar function.

Outer membrane 69-kDa protein: Brennan et al. (1988) identified a 69-kDa protein as a nonfimbrial agglutinogen present on all virulent strains of B. pertussis. Thomas et al. (1989) demonstrated antibodies specific for the 69-kDa molecule in human sera following disease or vaccination with whole-cell pertussis vaccine. It has also been shown that 16ug of 69-kDa outer membrane protein protects neonatal mice against lethal B. pertussis respiratory challenge (Shahin et al. 1990). The 69-kDa and FHA protein have been shown to have an "RGD" (Arg, Gly, Asp) cell binding sequence which functions in eukaryotic cell attachment. An "RGD" containing peptide constructed from the sequence of 69-kDa (pertactin) was able to inhibit attachment of CHO cells to pertactin (Leininger et al. 1990).

Toxins. B. pertussis produces many different toxins: pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, and tracheal cytotoxin, that have the ability to interfere with the clearing mechanisms of the lungs and the immune response.

Pertussis toxin: Pertussis toxin, a heat-labile protein, enters the bloodstream and exerts its systemic effects, e.g. leukocytosis, hypoglycemia, histamine-sensitivity, inhibition of migration and function of macrophages and probably inhibition of immune function. PT is an NAD-dependent ADP-ribosyltransferase (Katada and Ui, 1982) which affects the function of target cells by

catalyzing the transfer of ADP-ribose from NAD to an arginine of guanine nucleotide binding proteins (GTP binding proteins) and blocking the action of GTPase (Ui et al., 1984).

The increased amount of GTP results in the elevation of eukaryotic cellular adenylate cyclase. The A-protomer, composed of the S1 subunit, contains the ADP-ribosylating activity, and the B-oligomer, composed of the S2-S5 subunits, enables the binding of the A-protomer to various cell types and penetration of the cell membrane. It has been shown that the S2 and S3 polypeptides are able to bind to receptors on target cells (Witvliet et al. 1988; Nogimori et al. 1986). It has been suggested that the binding of the B-oligomer, more than the activity of the A-protomer, to eukaryotic cells causes the mitogenic activity associated with PT (Nogimori et al. 1984 and 1986). Tamura et al. (1983) demonstrated that the addition of PT to mouse or rat splenic cells increased the incorporation of [3H] thymidine into cellular DNA. The mitogenic action of the holotoxin was reproduced by the isolated B-oligomer portion of the toxin binding to the cells through the two dimers, D1 (S2 & S4) and D2 (S3 & S4). Therefore, the B-oligomer appears to have a dual function: (a) carrier of the A-protomer which is responsible for ADP-ribosylation of proteins in the target cell membrane and (b) as a mitogen of T-cells or other cells in which ADP-ribosylation is not involved. The literature contains conflicting reports on whether PT stimulates or

suppresses immune response. It appears that one must keep in mind that responses must depend on the strain of mice or other animals used, the kind and level of antigen administered, the route of inoculation, and the time at which PT is given relative to the antigen, all of which could result in the suppression of some classes of antibodies and stimulation of others, as well as the stimulation or suppression of some classes of T cells. Vogel et al. (1985) showed that doses of 250-500 ng/ml of PT inhibited the production of antibody plaque-forming mouse spleen cells in vitro, and Kumazawa and Mizunoe (1979) reported that the mitogenic dose of 1 ug/ml inhibited the in vitro response to sheep red blood cells. Doses of less than 1 ug/ml, however, appeared to stimulate antibody formation in vitro (Suzuki et al. 1978). The mitogenic activity on T-cells due to PT was shown to have a bimodal effect. At concentrations less than 0.4 ug/ml or more than 5 ug/ml there was no significant mitogenic action. The optimal condition was at a concentration of 2.5 ug/ml for 72 hours (Suzuki et al., 1978; Kong and Morse, 1977a,b; Ho et al., 1979; Fish et al. 1984). It was also shown that non-T-cells, or B-cells, were required for T -cells to respond to the mitogenic action of PT.

Adenylate cyclase toxin: Bordetella pertussis adenylate cyclase (AC) toxin is a calmodulin-activated enzyme which enters eukaryotic target cells and catalyzes the conversion of endogenous ATP into cyclic AMP

(Hewlett et al. 1989; Bellalou et al. 1990). The toxin is 216-kDa outer membrane protein of Bordetella that is probably transferred to target cells by phagocytosis of bacteria, or translocation from the bacterial membrane to the target membrane (Hewlett et al. 1988). It is suspected that it is a virulence factor of the bacterium (Hewlett et al. 1989; Bellalou et al. 1990). AC is released by the bacterium into the culture medium (Hewlett et al. 1976; Hewlett and Wolff, 1976; Weiss et al., 1986) and its activity has been shown to be increased by calmodulin (Wolff et al. 1980). These features suggest that this molecule might be a toxin. At least two forms of this adenylate cyclase have been identified; the first has been identified as an enzyme (70-kDa), and the second as a toxin (216-kDa) (Hanski and Farfel, 1985; Hewlett et al., 1986; Weiss et al., 1986). The enzyme portion was purified from the culture medium supernatant and was found to have a molecular weight of 70-kDa (Hewlett and Wolff, 1976). The 69-kDa nonfimbrial protein reported by Brennan et al., (1988) was also shown to be antigenically related to a protein previously correlated with the adenylate cyclase activity of Bordetella species (Novotny et al., 1985c). The majority of the adenylate cyclase toxin activity appeared to be bacterial cell-associated. Genetic data with Tn5 mutants suggest a common origin for these two forms of adenylate cyclase (Weiss et al., 1986, 1984, 1983).

Tracheal cytotoxin (TCT): Tracheal cytotoxin was isolated by Goldman et al. (1982). From the amino acid composition it appears that it may be derived from the bacterial peptidoglycan, which is a glycopeptide (921-dalton, disaccharide-tetrapeptide) of the cell envelope. The toxin has been shown to inhibit DNA synthesis in a cell culture of hamster tracheal epithelial cells and to cause cellular damage and ciliostasis to hamster tracheal epithelial cells. Data are consistent with the notion that TCT interacts with target cells via a specific receptor and interferes with a normal signal transduction pathway. (Goldman et al., 1990).

Heat-labile toxin (HLT) or Dermonecrotic toxin (DNT): This lethal toxin is cell associated, sensitive to 56^{0}C for 10 minutes, and causes necrotic lesions when injected subcutaneously into mice at low doses; at higher doses it is lethal (Livey and Wardlaw, 1984). Nakase and Endoh (1986) purified the toxin and found it had a molecular weight of 102-kDa with subunits of 30 and 24-kDa. HLT has pronounced vasoconstrictive activity, induces contraction of arterioles in the skin, perfused lungs and in smooth muscle-exposed arterial strips. It induces membrane damage and causes changes in permeability. B. pertussis HLT is antigenic in rabbits and guinea pigs, but not in mice. It may play a role in the initial stages of whooping cough by its vasoconstrictive action of peripheral arterioles in the lungs and respiratory tract. The inflammatory response may

be produced in combination with endotoxin (Nakase and Endoh, 1988).

Lipopolysaccharide (LPS) or endotoxin: The LPS or endotoxin of B. pertussis is distinguished from other gram negative organisms by having two distinct lipids, lipid A and lipid X, plus two different oligosaccharide chains (types I and II) (LeDur et al., 1980; Peppler, 1984). It has pyrogenic activity, promotes hypersensitivity to histamine, and induces nonspecific resistance to viral infections in normal and athymic mice (Munoz et al., 1978; Winters et al.,1985). Lipid X demonstrates the endotoxin activity and lipid A has potent adjuvant and antiviral activities in that it activates rabbit splenocytes and stimulates interleukin-1 secretion from human monocytes (Haeffner-Cavaillon et al., 1984; Haeffner-Cavaillon et al.,1982).

Other outer membrane proteins (OMP). Robinson and Hawkins (1983) reported 6 envelope proteins (30, 31, 33, 81, 86, & 90-kDa) specific to virulent B. pertussis that were reduced or absent in B. bronchiseptica, B. parapertussis and avirulent strains of B. pertussis. The envelope preparations showed protection in the mouse IC protection test; however, the protective potency was also shown to be correlated with the amount of LPF that each fraction contained. LPF alone was nonprotective, but in combination with envelope proteins the protective activity was restored. Wardlaw et al.,(1976) reported a correlation in virulent

strains between two outer membrane proteins of molecular weight 30-kDa and 28-kDa and the histamine sensitizing factor (HSF) and mouse protective antigen (MPA). Ezzell et al., (1981) demonstrated 10 major outer membrane proteins, 4 of which (98, 88, 30 and 28-kDa) were most affected by phase shift from virulent (phase I) to avirulent (phase IV) forms. Armstrong and Parker (1986a) showed that certain outer membrane proteins (OMP) were heat modifiable with regard to their migration behavior in SDS-PAGE. The modification was determined by various heat solubilization temperatures which resulted in altered electrophoretic mobility in SDS-PAGE, possibly due to heat induced SDS-protein interaction. They used surface radio-iodination, two-dimensional SDS-PAGE and Western immunoblotting with cell surface specific monoclonal antibodies to show that virulent strains had 5 heat modifiable exposed cell surface proteins. Two-dimensional gel electrophoresis revealed that two of these heat modifiable proteins (38-kDa and 18-kDA) migrated as higher molecular weight moieties when solubilized at low temperature (25^{0}C) and 3 proteins (91, 32, & 30-kDa) migrated as lower molecular weight species when solubilized at 25^{0}C; the latter three were found only in virulent B. pertussis. Two transposon-induced mutants that lacked the ability to produce hemolysis, dermonecrotic toxin, pertussis toxin, and FHA also lacked these three envelope proteins, thus confirming that virulence associated OMP were genetically regulated with other virulence traits (Armstrong and Parker, 1986b).

Growth in medium containing high levels of nicotinic acid caused phenotypic modulation and reduction of the 91, 32 and 30-kDa polypeptide fragments.

Endotoxin protein (EP), which refers to a select group of polypeptides in the outer membrane of gram-negative bacteria that are associated with the lipopolysaccharide endotoxin (LPS), are mitogenically active on B-lymphocytes and more potent than the LPS alone from which they were dissociated. Sultzer et al., (1985) demonstrated that EP stimulated interferon in mice, activated macrophages to be cytotoxic to tumor cells, acted like protective antigen against an infective challenge with the homologous organism and expressed adjuvant properties in CF-1 mice that had been immunized (IP) with graded doses of glutaraldehyde-inactivated cholera toxoid; or endotoxin protein from B. pertussis, Salmonella typhi or Vibrio cholerae. The most potent adjuvant activity was found in the B. pertussis EP which activated B-cells from non-responder mice in a polyclonal fashion. The endotoxin proteins are phenol-water co-extracted with the LPS and consist of three major peptides; 68, 65 and 30-kDa. These outer membrane proteins were not examined as separate components from the standpoint of immunobiological properties.

Another outer membrane protein of 40-kDa from a virulent strain of B. pertussis has been shown to be a porin protein which forms small, anion-selective channels in the lipid bilayer membrane. It has been postulated that the

permeability of the outer membrane may determine the organisms susceptibility to certain chemical compounds and antibiotics. Because this porin has been observed in all Bordetella strains, secretory antibody (IgA) reactive with the porin may be protective against colonization and infection, thus making the porin protein a possible vaccine component (Armstrong and Parker, 1986b).

Monji et al., (1986) found that purified outer membrane, containing mainly a 30-kDa protein, exhibited strong enhancement of IgM and IgG antibodies to Haemophilus influenza type b capsular polysaccharide, polyribosyl ribitol phosphate (PRP); however, PRP alone elicited only IgM class of antibody. Lipopolysaccharide (LPS) and leukocytosis promoting toxin (PT) components were removed from 4ml crude outer membrane preparation containing 4mg protein/ml by treatment with 2% Trition X-100, precipitated with 80 ml absolute ethanol overnight at 4^0C and then centrifuged at 9000 x g for 20 minutes. The precipitate was washed with distilled water, suspended in distilled water and dialyzed against phosphate buffered saline (PBS) for 3 days at 4^0C. The outer membrane preparation was further purified by DEAE-Sepharose 6B CL ion-exchange chromatography and Sephacryl S-300 filtration chromatography. The isolated OMP which enhanced PRP immunogenicity was characterized on SDS-PAGE. Protein profiles of the crude membrane fraction revealed two major bands at 30 and 62-kDa, and three minor bands at 72, 78, and 82-kDa. Membrane fractions partially

purified with 1% Triton X-100 and 50% ethanol demonstrated bands identical to crude preparation. Purified membrane from ion-exchange and gel filtration chromatography revealed that peak I (which showed the adjuvant effect with PRP) was essentially 30-kDa, and peak II (with no immunopotentiating activity) was composed of two major bands (60 and 62-kDa) and several minor bands of 24, 52, 73, 78, and 82-kDa. It was also shown that other biological activities of the outer membrane (OM) preparation, as well as the 30-kDa, did not stimulate antibody response to pneumococcal type VI polysaccharide nor did they induce protection in mice against IC challenge with B. pertussis. It was speculated that lack of protection was probably due to lack of PT in the preparations. The purified LPS or pertussis toxin when combined with PRP did not exhibit adjuvant activity for the polysaccharide antigen.

PERTUSSIS VACCINES

Brief history of immunization. Attempts to produce a whooping cough vaccine began soon after Bordet and Gengou (1906) isolated the causative organism. The first indication of protective efficacy was reported by Madsen (1925, 1933) in the Faroe Islands during the epidemic of 1923-1924. Sauer (1933, 1937), Kendrick and Eldering (1936, 1939) and Leslie and Gardner (1931) were some of the early pioneers in the development of whole-cell pertussis vaccines. It was Leslie and Gardner's (1931) work that

demonstrated phase changes occurred *in vitro* during the growth of *B. pertussis* and that the smooth phase I or virulent stage was the only form suitable for vaccine production. More recent work has shown that phase change is characterized by the loss of virulence factors (Weiss and Falkow, 1984; Wardlaw *et al.*, 1976; Wardlaw and Parton, 1979; Dobrogosz *et al.*, 1979; Hewlett *et al.*, 1979; Robinson and Hawkins, 1983; Goldman *et al.*, 1985; Weiss and Hewlett, 1986). Development of procedures to control and standardize the growth, detoxification, standardization of cell suspensions with opacity reference preparations, measurement of potency against a reference vaccine by IC challenge in mice and mouse weight gain test for measurement of toxicity, have been important in the production of whole-cell pertussis vaccines. Current regulations controlling the production of pertussis vaccines were described by the Expert Committee on Biological Standardization (World Health Organization, 1979). The regulations specify the appropriate starting seed pools, strict adherence to established protocols, tests for purity, potency and safety at specific steps in production, and accurate record keeping. Suitable strains of *B. pertussis* of known origin, history and characteristics are essential, plus the final finished vaccine should contain agglutinogens 1,2 and 3. To ensure consistent potency, each vaccine is standardized against a reference vaccine, a national preparation which has also been standardized against an International Standard for

Pertussis Vaccine. The current international reference, which is the second such reference, was established in 1980 and is kept at the Statens Seruminstitut in Copenhagen. It is a freeze-dried preparation containing 46 International Units of protective potency. By comparing the relative potency of test vaccines with the reference vaccine (in the mouse potency assay), the protective activity of the test vaccines can be expressed in International Units per single human dose.

With the decline in the incidence of whooping cough as a result of vaccine programs, the public became concerned that adverse side effects of the vaccine were of greater risk than the disease (Miller et al., 1982). Local reactions (e.g., redness, tenderness, swelling) and fever are seen frequently within 12 to 24 hours following vaccination. The more severe reactions associated with whole-cell pertussis vaccine have been encephalopathic in nature, resulting in permanent brain damage and death. In the 1970's and 1980's the publicity surrounding these severe reactions lead to a decline in use of vaccine in the United Kingdom and Japan and subsequently large epidemics ensued. As a result of the controversy surrounding the use of pertussis vaccine in the United Kingdom, a National Childhood Encephalopathy Study (NCES) was established in 1976 to determine whether pertussis vaccine did indeed cause brain damage and if so to what degree. The results of 1000 cases over a 3 year period were published by Alderslade et

al., (1981). A case was defined as acute neurological illness in a 2 to 36-month old child that required hospitalization, and encephalopathy was defined as a case with residual effects after 1 year. In previously normal children the estimated risk of neurological complications from Diphtheria-Tetanus-Pertussis (DTP) vaccine was 1 in 110,000 immunizations and the risk of encephalopathy was 1 in 310,000 immunizations. The conclusions of the NCES were: that most cases of neurological involvement were due to causes other than immunization; cases occurring within 72 hours of vaccination did so more frequently than expected by chance alone; most children expressing neurological complications made a complete recovery; and that similar cases occurred after Diphtheria-Tetanus (DT) vaccine alone, which indicated that permanent damage as a result of pertussis vaccine was a rare event. It was noted that no causal relationship could be established between DTP immunization and serious neurological illness; but, for some children there was evidence that a link may exist. Both in the United States and the United Kingdom it has been confirmed that the use of whole-cell vaccine effectively protects against pertussis, a disease that also causes brain damage, and that the advantages of prevention out weigh the risks associated with its use (Joint Committee on Vaccination and Immunization, 1981; Immunization Practices Advisory Committee, 1981).

The disease is still of world wide importance as a cause of death and disability. While improved child health care and socioeconomic conditions have reduced the impact of the disease, it is also clear that vaccination is still very important in continued control. Due to these ever present concerns, efforts to develop acellular pertussis vaccines have been aimed at the characterization and purification of selected protein components of the organism. An acellular vaccine developed in Japan contains FHA, formalin detoxified toxin and other proteins obtained from the supernatant of the organism, combined with Diphtheria and Tetanus toxoids and adsorbed to aluminum hydroxide/aluminum phosphate (Sato et al., 1984). The potency of the component vaccine is determined by the mouse potency test and is comparable to existing whole-cell vaccines. There are several such acellular vaccines in use at this time.

AT the present time a component, acellular, pertussis vaccine is being developed by the Michigan Department of Public Health. This vaccine contains equal amounts of pertussis toxoid (PTx) and filamentous hemagglutinin (FHA) combined with Diphtheria and Tetanus toxoids adsorbed to aluminum hydroxide. During the purification of the pertussis toxin component, it was noted that an eluate obtained from an affinity column contained component(s) that increased the protective ability of purified pertussis toxoid. The purpose of the research reported in this work was to identify and characterize any protective proteins or

other components within this fraction that might account for the associated protective or immunoenhancing capability demonstrated with purified pertussis toxoid.

MATERIALS AND METHODS

History of Bordetella pertussis strain 18334. Bordetella pertussis strain 18334 was isolated in 1944 from a whooping cough patient by Dr. Pearl Kendrick and Dr. Grace Eldering of the Michigan Department of Public Health Laboratory in Grand Rapids, Michigan. It was initially grown on Bordet-Gengou (BG) agar, resuspended in sterile skim milk and freeze dried. The strain was reconstituted, subcultured three times and lyophilized in 1946, 1948 and 1950. Vials from lyophilization #4 (1950) were sent to the Lansing, Michigan laboratories of the Michigan Department of Public Health where they were reconstituted, subcultured three times and lyophilized a fifth time. Lyophilization #5 was again reconstituted, subcultured three times and lyophilized in 1975 and 1988. Vials of the sixth lyophilization (1975) have been set aside and designated as the master seed stock of Bordetella pertussis 18334.

Acellular component vaccine. The growth and maintenance of the organism, and the procedures for isolation and purification of filamentous hemagglutination factor (FHA) and lymphocyte promoting factor (LPF), also called pertussis toxin (PT), have been previously reported (Shih et

al.,1986). Spheroidal hydroxylapatite (BDH Chemicals Ltd., Poole, England) chromatography is used in the initial separation and purification of FHA and crude pertussis toxin. Crude LPF or pertussis toxin is further purified with fetuin-sepharose affinity column chromatography. CNBr-activated Sepharose 4B (Pharmacia, Piscataway, New Jersey) is coupled to fetuin according to the procedures described in the Pharmacia protocol. The semi-purified toxin is mixed with the fetuin-sepharose, stirred for 24 hours at 4^0C, and then poured into a 2.5cm x 20cm column (Bio-Rad Laboratories, Rockville Centre, N.Y.). The initial effluent from this column (1.5 to 2.0 liters) is collected and has been designated as "Peak K".

Characteristics of Peak K. From preliminary studies, it appeared that some component(s) in the K fraction had immunoenhancing and/or protective properties against pertussis. It was noted that semi-purified pertussis toxoid gave better protection than the highly purified LPF toxoid against pertussis challenge in mice. The K fraction was applied to a Sephacryl S-300 (Pharmacia, Piscataway, N.J.), 2.2cm x 85cm molecular exclusion column and two additional peaks, designated Ks-1 and Ks-2, respectively, were resolved. Of these two fractions, Ks-1, more than Ks-2, appeared to increase the protective activity of pertussis toxoid in mice challenged intra-cranially (IC) with live pertussis cells.

Concentration methods. Bulk material to be analyzed was concentrated by various methods. The DC-2 Amicon (Amicon Div., W.R. Grace & Co., Danvers, MA.) dialyzer-concentrator system with 10,000 MWCO cartridge was washed with distilled water, 100ppm sodium hypochlorite, 0.5% formalin and 0.1N NaOH for several cycles at a flow rate between 100 and 190 ml per minute. The unit was allowed to stand over night in 0.1N NaOH and then equilibrated with 0.1M sodium phosphate/0.5M NaCl pH 7.0 prior to use. A total of 6 liters, comprised of a pool of 7 sublots of peak K, was concentrated to 5% of the original volume.

Individual sublots (800 to 1000ml) were first dialyzed against 0.1M Tris with 0.1% sodium dodecylsulfate (SDS) at pH 7.4 before being placed in the Virtis Freeze/Dryer Model #25SRC (Virtis Co.,Gardiner, N.Y.,). Vials were reconstituted to 5% of their original volume in 0.1M sodium phosphate/0.5M NaCl, ph 7.4.

To remove the organic solvent, RP-HPLC fractions were dried to a volume of approximately 2ul in a Speed Vac Evaporator/Concentrator (model SVC 100 or 200H Savant Instruments, Farmingdale, N.Y.), depending on the fraction volume. The protein was resuspended in either 20ul of 1X sample buffer (70mM Tris pH 6.8, 11.4% v/v glycerol, 3% SDS, 0.01%BPB, 5% v/v 2-ME, Milli-Q water to volume) for direct application to SDS-PAGE, phosphate buffered saline (PBS) with 10% glycerol for use in vivo or in vitro, or 0.01M Tris (ph 7.4) with 0.1% SDS, 1mM phenyl methyl sulfonyl fluoride

(PMSF) (Sigma Chemical Co. St. Louis, MO.) or 1ug/ml leupeptin (Boehringer Mannheim, Indianapolis, Ind.) for Western blotting or electroelution from gels.

Chromatographic procedures. Gel filtration, or size exclusion chromatography based on the relative size of protein molecules, was used to separate crude peak K into two fractions, Ks-1 and Ks-2, respectively. A Sephacryl S-300 column (Pharmacia), with a fractionation range of 10,000 - 1.5 x 10^6 daltons and consisting of cross-linked dextran beads, was poured into a 2.2cm diameter glass column to a level of 90cm. The column was washed with 0.1N NaOH and equilibrated with 0.1M sodium phosphate/0.5M NaCl, pH 7.0. Approximately 30ml of crude 20X concentrated peak K containing an average of 50 mg protein was centrifuged for 10 minutes at 3000 rpm, layered on the surface of the gel and allowed to percolate through the matrix at a flow rate of 35 ml/hour. Four ml fractions were collected and protein monitored at 280nm in a Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Ca.). Graphics plot of protein analysis at 280nm was done by Sigma Plot version 3.1 (Sigma Chemical Co.).

A Tosohaas TSK G3000SW silica based gel 7.5mm x 300mm column (P.J. Cobert Associates, Inc., St. Louis, Mo.) with a fractionation range of 1 - 300kDa was used with the HPLC Waters 600 multisolvent delivery system, System Gold Programmable Beckman Detector Module 166 and WISP 710B

automatic fraction injector (Millipore Corporation, Bedford, Ma.). This high performance exclusion type material was run at a flow rate of 0.6ml/min. in .05M sodium phosphate/0.1M NaCl, pH 6.5. The elution time of unknown fractions was compared to a set of known standards (Sigma Chemical Co.). The molecular weight of peptides within fractions from various sublots was determined by linear regression analysis of elution volumes.

Analytical and preparative Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) columns were used to resolve or screen peptides in Ks-1 and Ks-2 and for in vivo and in vitro studies. The organic buffers used were as follows: Buffer A: 0.1% trifluoroacetic acid, pH2-3, (Pierce, Rockford, Ill.) in 0.2u filtered, helium sparged Milli-Q water (Millipore Corp.) and Buffer B: acetonitrile (Burdick & Jackson, Baxter Scientific Products, Romulus, MI.), isopropyl alcohol (Baxter Scientific Products), 0.2u filtered, helium sparged Milli-Q water in a ratio of 60:25:15 with approximately 890ul TFA added to balance absorbance in the analytical columns or helium sparged acetonitrile with the addition of 0.1% TFA for the preparative column. Analytical columns 2mm x 250mm and 4.6mm x 250mm C-18 Vydac 218TP54 (The Separations Group, Hesperia, Ca., 92345) were used initially to determine gradient conditions for the preparative separation. The 22mm x 250mm C-18 Vydac 218TP152022 preparative column was used with the Waters Prep LC 3000 System (Millipore Corp.,

Bedford, MA.) and low pressure mixing at a flow rate of 30 ml/minute. Vydac C-18 is recommended for small peptides which are better retained due to increased interaction with the longer alkyl chain bonded to the silica-based support.

Polyacrylamide gel electrophoresis and electroblotting.

Samples from RP-HPLC were analyzed by discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedures of Laemmli (1970). Gels were stained with either silver nitrate (Morrissey, 1981) or Coomassie Brilliant Blue R-250 (Sigma Chemical) according to the procedure manual published with the Bio-Rad Mini-Protean II Dual Slab Cell (Bio-Rad Laboratories, Rockville Centre, N.Y.). In brief, 0.1% Coomassie, dissolved in 40% methanol and 10% acetic acid, was used to stain gels for 30 minutes followed by destaining with 40% methanol/10% acetic acid to remove background. Peptides transferred from mini-gels to ProBlott (Applied Biosystems, Foster City, Ca.) or Immobilon P (Millipore Corp.) were stained with Coomassie for 1 minute (ProBlott technical bulletin No. 400994 and Yuen, et al., 1990).

Electroblotting transfer buffer containing 25mM Tris, 192mM glycine and 20% methanol was preferred to a CAPS buffer (3-[cyclohexylamino]-1-propanesulfonic acid) with 10% methanol. Various transfer parameters were tried and the method which appeared best for transfer of high molecular

weight peptides was 150 mAmps for 16-24 hours in the Bio-Rad Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Labs.). Except for these modifications, the electroblotting procedure was done according to the method in Applied Biosystems technical bulletin No. 400994 and Yuen, et al., 1990).

Immunostaining of blotted membranes was modified to reflect the need for mouse monoclonal antibody-antigen reaction to be amplified with an biotin-avidin step in the staining procedure. To prevent nonspecific binding, membranes were initially blocked for 45 minutes with 5% non-fat dry milk in 25mM Tris/HCl in 0.5 M NaCl, pH 7.5, (TN buffer), followed by normal serum in 0.1% (v/v) Tween 20 in Tris-buffered saline (100mM Tris, 0.9% NaCl, pH 7.5) for 30 minutes according to the method described in the Vectastain ABC Mouse IgG and IgM and Rabbit IgG horseradish peroxidase kits (Vector Laboratories, Burlingame, CA.). The membranes were probed with the primary antibody of interest and followed by a biotinylated affinity purified anti-immunoglobulin. This complex was then detected by an avidin/biotinylated horseradish peroxidase reagent and visualized by reacting with a substrate solution. Substrate solutions using diaminobenzidene (DAB) or 4-chloro-1-naphthol (4CN) did not prove to be as suitable as a tetrazolium substrate (Taketa et al., 1986). The tetrazolium substrate is composed of the following: 100mg

NADH, 100ul phenol, 34ul H_2O_2, 17mg Nitro-Blue Tetrazolium in 50ml 50mM sodium phosphate pH 7.0.

Monoclonal antibodies. The 9D4 monoclonal antibody specific for the 216kDa adenylate cyclase holotoxin and its break-down products was obtained from Dr. Erik L. Hewlett (University of Virginia, Dept. of Medicine, Charlottesville, Virginia). The monoclonal antibodies designated BPE3, (specific for the 69kDa outer membrane protein), BPF2 (anti-fimbriae 2), BPC10 (anti-LPS-A), and BPG10 (anti-fimbriae 3/6) were received from Dr. Charles R. Manclark (Laboratory of Pertussis, Center for Biologics Evaluation and Research, Bethesda, Md.). These monoclonals were from ascites fluid and used in the Western Blot procedure at a dilution of 1:1000. Monoclonal anti-FHA (P12H3) and LPF-S1 (B2F8/A4) were received from Dr. Charlotte Parker, (University of Mo., Columbia, Mo.) and used at 1:1000 in Western Blot. Also received from Dr. Parker were experimental culture supernatants 1F11 and P8E7 (anti-91-kDa) and P1E8 and 1D7 (40-kDa porin protein) used undiluted in Western blot.

Electroelution of peptides from SDS-PAGE. Peptides of molecular weights 216-kDa, 125-kDa, 100-kDa, 90-kDa, 69-kDa, 43-44-kDa doublet, and 30-31kDa doublet were sliced from polyacrylamide gels and eluted in the Bio-Rad Electroeluter Model 422 (Bio-Rad Labs.) according to published procedures (Hunkapillar et al., 1983) and the Bio-Rad procedure manual. Gel slices were placed in glass tubes equipped with 3500

MWCO dialysis membranes and eluted for 5-6 hours at 60 mAmps. Eluted proteins were washed from the membrane-gasket chamber with 0.2ml 0.01M Tris, 0.1% SDS and 1ug/ml leupeptin.

Protein determination assays. Protein analysis was done by the Lowry Method (Lowry et al., 1951), the Bio-Rad microassay based on the Bradford dye binding assay (Bradford, 1976) and direct measurement with the Beckman DU 64 Spectrophotometer equipped with Soft-Pac Modules. The Soft-Pac program determines the concentration of protein and nucleic acid in micrograms/ml by the Warburg and Christian absorption equation (Warburg and Christian, 1942).

Animals. Female mice (11-14g) derived from a population of Swiss Webster CRL.CFW(SW)BR from Charles River Laboratories and maintained at the Michigan Department of Public Health (MDPH) were used in these studies.

Rabbits (8-10 weeks of age) were from a Great Lakes strain of pedigreed New Zealand white rabbits maintained by a Class A Federal Licensed commercial breeder (Dettmer's Bunny Patch, Carson City, MI.). The rabbits have been shown to have a history of Bordetella bronchiseptica.
Sera from the rabbits used in these studies were sent to Dr. David Bemis, University of Tennessee, Knoxville, Tn., in an attempt to determine the antibody status to Bordetella bronchiseptica.

English, short-haired, albino, outbred guinea pigs (strain Mdh:(SR(A)) were produced and maintained in the MDPH

animal breeding facilities. Guinea pigs of either sex between 275 -325g were used in these experiments. These animals were anesthesized with 0.3ml xylazine (5mg/kg body weight)-ketamine (35mg/kg body weight) prior to obtaining blood samples by cardiac puncture.

The mice and guinea pigs can be classified as specfic pathogen free (SPF). Routine testing is done for Sendai virus, Pneumonia virus of mice, mouse Polio virus, Reovirus, Mycoplasma pulmonis, mouse Hepatitis virus, Lymphocytic choriomeningitis virus and Encephalitozoon cuniculi. Guinea pigs are also checked for Streptococcus, and Bordetella by nagopharyngeal washes. They have been found to be free of ecto- and endo-parasites (B. Kintner, DVM, personal communication). However, infection with adventitious agents could not be ruled our since none of the experiments were conducted in isolators. There is no scheduled testing program for the rabbits; therefore, they are considered to be conventional. They are held and observed for one week prior to use.

Functional Assays. The rapid fluorescent focus inhibition test (RFFIT) as described by Smith et al., (1973), was used to determine the antibody titer of sera from guinea pigs vaccinated with a 1X rhesus diploid rabies vaccine (RDRV), produced by the Michigan Department of Health (Burgoyne et al., 1985) either alone or in combination with RP-HPLC purified fractions. Sera were tested with serial 5-fold dilutions, beginning with a 1:5 dilution, and mixed with a

constant volume of Challenge Virus Standard (CVS-11) strain of rabies virus (Kissling, 1958) that produced between 10 and 20 FFD_{50} in the virus titration. The virus-antibody dilution mixture was placed in a 35°C incubator with 5% CO_2 for 90 minutes. Each dilution per sample was added to a well of a 48-well cluster dish (Costar, Cambridge, MA.) containing 2 x 10^5 baby hamster kidney cells, clone 13 (American Type Culture Collection, Bethesda, MD.). Growth media consisted of Modified Eagle's Medium (MEM), supplemented with 10% heat-inactivated fetal calf serum, 10% tryptose phosphate broth and 100 units/ml penicillin-streptomycin. After 24 hours cells were washed, fixed with methanol and stained with fluorescein labeled anti-rabies globulin (BBL Microbiology Systems, Becton-Dickinson & Co., Cockeysville, Md.).

ELISA. The enzyme-linked immunosorbent assay (ELISA) (Engvall & Perlman, 1972) was modified to quantitate pertussis toxin (PT) and FHA antibodies (Manclark et al., 1986) in sera from rabbits immunized with purified pertussis toxoid (PTx), FHA and peak Ks-1 fractions. Rabbit anti-LPF antibody was determined by coating Dynatech Immulon-2 flat bottom 96 well dishes (Dynatech Laboratories, Inc., Chantilly, Virginia 22021) with toxin (2ug/ml) diluted in incubation buffer (bovine serum albumin and polypropylene glycol), inoculated 50ul/well and incubated for 1.5 hours at 35°C. Plates were washed 10 times with PBS/Brij-35 solution in a Biotek Microplate Autowasher EL403H (Biotek

Instruments, Inc. Winooski, Vermont). Anti-LPF polyclonal rabbit antiserum was diluted in 8-2 fold serial dilutions beginning with 1:500, normal rabbit serum at 1:250, and unknowns in 8-2fold serial dilutions starting at 1:250 in PBS/Brij-35/1% FCS. Fifty ul of each dilution were added to the appropriate wells and incubated for 1.5 hours at 28°C, followed by 10 washes. Goat anti-rabbit alkaline phosphatase conjugate (Kirkegaard & Perry Labs., Inc., Gaithersburg, Md.), diluted 1:1000 in PBS/Brij/FCS, was added (50ul) to each well and incubated 1 hour at 28°C, washed 10 times and reacted with substrate, (100ul/well) 1mg/ml para-nitrophenyl phosphate (Sigma 104 tablets)and incubated 30 minutes at 35°C. The reaction was stopped with 10ul/well 5N NaOH and read at 405nm on a Maxline Microplate Reader (Molecular Devices Corporation, Menlo Park, Ca.). The data were analyzed by a basic language computer program from U.S. Food & Drug Administration based on linear regression and parallel line bioassay (Manclark et al., 1986; Brownlee, 1965). Unitage data for each test sample and the reference are plotted as $\log_{10}$ dilution vs. optical density or absorbance reading at 405nm. The linear portion of the reference and test sera dose response curves were redrawn parallel using a common, pooled slope. The reference antibody for these data was set at 100 units. Antibody levels for FHA were determined by the same procedure except that wells were initially coated with 50ul rabbit anti-FHA lot#1 diluted 1:6000 in PBS, pH 7.4,

incubated 1 hour at 35°C, washed 10 times and blocked with 200 ul/well with fresh 2% BSA (Sigma Chemical, Fraction V). Plates were incubated 1 hour at 35°C before the FHA antigen, diluted 1:250, was added and then incubated again for 1.5 hours at 35°C.

Gel purified peptide bands were analyzed for the presence of LPF and FHA contaminating antigens by direct ELISA in which mouse monoclonal B2F8/A4 (anti-LPF), diluted 1:32 in PBS/0.1%Brij/1%FCS, and P12H3 (anti-FHA) were used. (Monoclonal antibody was obtained from C. Parker, University of Missouri, Columbia, MO.) Affinity purified alkaline phosphatase-conjugated, goat, anti-mouse IgG was applied at 50ul/well and incubated 1 hour at 28°C. The reaction was stopped with 5N NaOH and read as before.

Lymulus Amoebocyte Lysate (LAL) TEST. Reverse Phase-HPLC fractions and gel purified fractions were also analyzed for the presence of endotoxin by the LAL test (Levin & Bang, 1968). Pyrotell (LAL lot #96.85.332, Associates Cape Cod, Inc., Falmouth, MA.) was used to determine the amount of endotoxin based on the sensitivity of 0.25 endotoxin units (EU)/ml using E. coli endotoxin as standard. LAL powder was reconstituted with 5 ml Water For Injection (WFI), aliquoted and stored at -20°C. Sephacryl peaks Ks-1 and Ks-2 were used at 10^{-4} as positive controls, and WFI and 0.1M sodium phosphate/0.5M NaCl buffer as negative controls. Test samples were diluted in serial 10-fold dilutions. Fractions were tested by transferring 0.1ml of each dilution to

another tube, adding 0.1ml LAL gel clot and incubating in a 37^{0}C water bath for 60 minutes. The presence of a gel clot in the test samples was indicative for endotoxin (sensitivity to picogram level). Federal guidelines state that 0.1ng/ml of endotoxin given in a dose of 10ml/kg of a rabbit's body weight is the level that will elicit a minimum pyrogenic response in 50% of the rabbits injected. Converted to endotoxin units (EU) this would equal 0.5 EU/ml based on the U.S. Standard Endotoxin lot EC-2. The guidelines limit concentration of endotoxin to 2.5 EU/ml (0.5ng/ml) for parenteral drugs (Hochstein, 1981).

Gel electrophoresis purified fractions were tested by using Endotect (Schwarz/Mann Biotech. Div. of ICN Biomedicals, Inc., Cleveland, Ohio), a gelation reaction of LAL in capillary tubes. The level of sensitivity for this test is 0.06 to 0.1ng/ml endotoxin. In both methods the endotoxin activates an enzyme in LAL which subsequently decomposes coagulative protein in LAL resulting in gel formation. The positive Endotect capillary reaction occurs by dissolving the dried LAL with endotoxin containing water resulting in immobility of the reacted liquid. Sample capillary tubes are dipped into the test liquid until the liquid reaches a specified level. The tube is then inverted to allow the liquid to contact the endotoxin reagent and then mixed by vertical rocking and placed in a 37^{0}C incubator for 60 minutes. Immobility constitutes a positive test.

Chinese Hamster Ovary Assay (CHO). The CHO cell line (ATCC #CCL 61) was used to assay for pertussis neutralizing antibodies in sera from rabbits immunized with purified PTx, FHA and two different molecular weight peptide fragments obtained by eluting the bands from SDS-PAGE containing Peak "K" from sublot 60. The CHO cell assay (Gillenius et al., 1985) was done according to the guidelines from FDA, Center for Biologics Evaluation and Research, Laboratory of Pertussis, Serology Section, (Meade, pre-print, 4-30-90). Cells were first thawed, centrifuged to remove cryogenic additives as recommended by ATCC, resuspended at 1:4 dilution in a $75cm^2$ Corning tissue culture flask (Corning Glass, Corning, N.Y.) with Ham's F-12 media supplemented with 10% FCS and incubated at 37^0C in a CO_2 humidified chamber until a confluent monolayer was formed (approximately 4 days). Growth media was removed and the cell monolayer was rinsed and trypsinized with 0.25% trypsin solution in Dulbecco's balanced salt solution without calcium and magnesium ions. The flask was placed in a 37^0C incubator and monitored for cell detachment. Ham's F-12 with 10%FCS was then added to the flask to dissociate the cells into suspension by gentle pipetting action, followed by centrifugation for 10 minutes at 1000 rpm. Cells were counted by using a vital stain, e.g. Trypan Blue (Gibco, Grand Island, N.Y.) in a hemocytometer and diluted to 1 x 10^5 cells/ml. The assay is based on the ability of PT to cause a clustering morphology of the cell monolayer.

Neutralizing activity of sera is determined by making 11-2 fold serial dilutions of test serum and adding a constant amount of toxin (2ng/ml) to each appropriate well of a 96 well culture plate. The serum-toxin mixture is incubated 3 hours at 37^0 in 5% CO_2 after which 100ul of the cell suspension is added to each well and the plate again incubated for 48 hours at 37^0C. Neutralization titers are reported as the highest final dilution of a test serum that fully neutralized the pertussis toxin clustering activity. The amount of toxin added to each well should contain 4 cytopathogenic units (CPU). One CPU equals the lowest amount of toxin that causes a cytopathogenic response or clustering in the pertussis toxin titration.

Mouse protection tests. In an attempt to identify peptides that would enhance the protective activity of the MDPH acellular vaccine, the mouse protection test (Kendrick et al., 1947) was used to evaluate semi-purified RP-HPLC fractions and PAGE fractions. Female CRL-CFW mice (11-14g) were vaccinated by the intraperitoneal (IP) route, 0.5ml/dose/mouse, with acellular vaccine alone, LPF + FHA alone, or in combination with selected fractions at various concentrations. The mice were challenged 3 weeks later with live pertussis cells (1×10^{-4} opacity units/0.03ml IC) and terminated 14 days post challenge. The pertussis vaccine potency test was performed and calculated by the logit statistical method of Wilson and Worcester (Worcester and WWilson, 1943). The relative potency of the test vaccine is

determined by dividing the ED50 of the reference vaccine into the ED50 of the test vaccine and multiplying by 8 units/ml of reference X 1.5ml (total human dose of the product).

Immune response. Cell-mediated and humoral immune response to acellular factors was examined by immunizing rabbits at 8-10 weeks of age with LPF and FHA alone or with fractions, in Freund's complete adjuvant (Difco, Detroit,MI.), 1ml/ popliteal lymph node. Peripheral blood lymphocytes (PBL) were separated from 8-10ml whole blood in 1.5ml acid-citrate-dextrose (ACD) at various times post immunization. Either Lymphoprep (Nycomed Pharma, Oslo, Norway; Distributed by Accurate Chemical & Scientific Corp., Westbury, N.Y.), 1.077 specific gravity or Ficoll (Sigma Chemical)/ Hypaque, 75% (Winthrop Laboratories, Div. Sterling Drugs, Inc., New York, N.Y.), specific gravity of 1.075 was used as a separation medium. The blood was diluted with 2 volumes of Iscove's media (Gibco) at pH 6.5 to inactivate platelets and 5ml layered on 4ml of separation medium in a 15ml centrifuge tube. The tubes were centrifuged at 800g for 20 minutes. Cells at the interface were transferred to 50ml centrifuge tubes (Corning, Corning, N.Y.), washed with Dulbecco's PBS (Gibco) (without calcium and magnesium ions) supplemented with 0.1% normal rabbit serum, centrifuged at 400g for 10 minutes, followed by additional washes in Dulbecco's PBS with 5% normal rabbit serum (NRS). These lymphocytes were either used without

further separation or were resuspended in warmed (37^0) Iscove's pH 7.2 with 20% NRS, counted and applied to a 30ml syringe packed with nylon wool that had been previously rinsed with 5-10ml of warmed medium. The columns containing cells (5×10^7/ml) were placed in a 37^0C incubator for 45 minutes. Non-adherent T-cells were collected in a 50ml centrifuge tube by washing and eluting the columns with 60 ml warm media. The cells were centrifuged at 200g for 10 minutes, resuspended in Iscove's with 15% NRS, 2mM glutamine, ß-mercaptoethanol (10^{-5}M) and 100 units pen.-strep. Adherent B-cells and macrophages were recovered by adding cold Dulbecco's PBS (without calcium/magnesium ions) to the column and forcibly pushing fluid out with the plunger. These cells were applied to a 100mm cell culture dish for 1 hour at 37^0C to remove most of the macrophages. Non-adherent cells were washed with Dulbecco's PBS (without calcium and magnesium ions), counted and resuspended in supplemented Iscove's with 15% NRS. An *In vitro* B cell assay was done according to a method described by Wiertz, *et al.*, (1989). Peripheral blood lymphocytes (2×10^6) were cultured in 12 x 75mm tubes in the presence of medium alone or antigen dilutions (2000ng to 1ng) in a final volume of 5ml media per tube. After 48 hours at 37^0C in 5% CO_2, supernatants were removed, pellets were washed 2 times with serum free Iscove's, resuspended in 1ml of supplemented Iscove's with 10% NRS, and incubated for 10 days at 37^0C in

5% CO_2. Supernatants were harvested and assayed for antibody production by ELISA.

T cells from the nylon wool columns were analyzed in a proliferation assay (Bradley, 1980; Wiertz et al., 1989). Each lymphocyte suspension was diluted to contain 1 x 10^6 cells/ml and 0.1ml inoculated/ appropriate well of a 96 well, round bottom microplate (Gibco) in the presence of 100 ul media or antigen concentrations of 2500ng to 5ng/ml. Mitogen ,e.g., phytohemagglutinin (PHA-M), concanavalin-A (Difco, Detroit, MI), and lipopolysaccharide (LPS) from E. coli (Sigma Chemical, St. Louis, Mo.) stimulation of lymphocytes was used as a positive control. Plates were incubated for various times at 37^{0}C in humidified air with 5% CO_2. Mitogen plates were harvested at 24, 48, and 72 hours and antigen plates were harvested at 48, 72 and 96 hours. At 6 and 24 hours prior to harvest, cells were checked for viability before being pulsed with 1 uCurrie/well [methyl-3H] thymidine (Amersham Corp., Arlington Heights, Ill., Sp. Act. 2 Ci/mMol, concentration 1mCi/ml; Dupont Corp., Wilmington, Del., Sp. Act. 2 Ci/mMol, concentration 1mCi/ml) or 1uCi/well [methyl-3H] deoxythymidine 5'-triphophate, tetrasodium salt (Dupont Corp., Wilmington, Del.), Sp. Act. 80 Ci/mMol, concentration of 2.5 mCi/ml in 10mM tricine-NaOH buffer, pH 7.6). Lymphocytes were harvested onto Mini-Mash II glass fiber filter strips (grade 934 AH) with a Mini-Mash II cell harvester (Whitaker M.A. BioProducts, Walkersville, Md.).

Filter discs were placed in Beckman Poly-Q scintillation vials with 4 ml liquid scintillation cocktail (Bio Safe II, biodegradable, Research Products International) and counted in the Beckman Model LS 1801 liquid scintillation counter. Results from triplicate wells were averaged and a stimulation index determined by calculating the ratio of the mean counts per minute (cpm) of the experimental wells divided by the mean cpm of control cultures containing media.

Amino Acid Sequencing. The 69kDa peptide was electroblotted on to ProBlott for direct sequencing in the Applied Biosystem (Foster City, Ca.) 477A Protein Sequencer with pulsed liquid chemistry. The system integrates Edman chemistry, PTH amino acid analysis and computer aided sequence analysis.

RESULTS

Characteristics of Ks-1 and Ks-2 subfractions. Peak "K", the eluate obtained during the purification of pertussis toxin was further resolved by gel filtration chromatography on a sephacryl S-300 column (Figure 1) into two subfractions, Ks-1 and Ks-2. Preliminary data, as well as data shown here, indicated that some component(s) in Ks-1, more than Ks-2, increased the mouse protective ability of purified pertussis toxoid. The chromatograms from Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) with a C-18 analytical column and 90 minute gradient along with associated discontinuous polyacrylamide gel (SDS-PAGE) pictures are shown in Figures 2 and 3. Ks-1 contained higher molecular weight peptides that appeared around 62.5% B-buffer. Ks-2 contained lower molecular weight peptides, especially noticeable was a heavy band at approximately 30-kDa (50% B-buffer). The data in Table 1 represent the parameters examined in these fractions. The chromatograms from the larger preparative RP-HPLC column and their respective SDS-PAGE pictures are shown Figures 4 and 5. The larger column configuration and increased flow rate show the appearance of the higher molecular weight bands in Ks-1 at approximately 43% B-buffer. The 30-kDa band in the Ks-2

Figure 1. Resolution of peak "K" on a 2.2cm x 84cm Sephacryl S-300 gel filtration column. Four ml fractions were collected and the protein content analyzed at 280nm. Average flow rate = 35ml/hr. Bed volume = 319.3ml

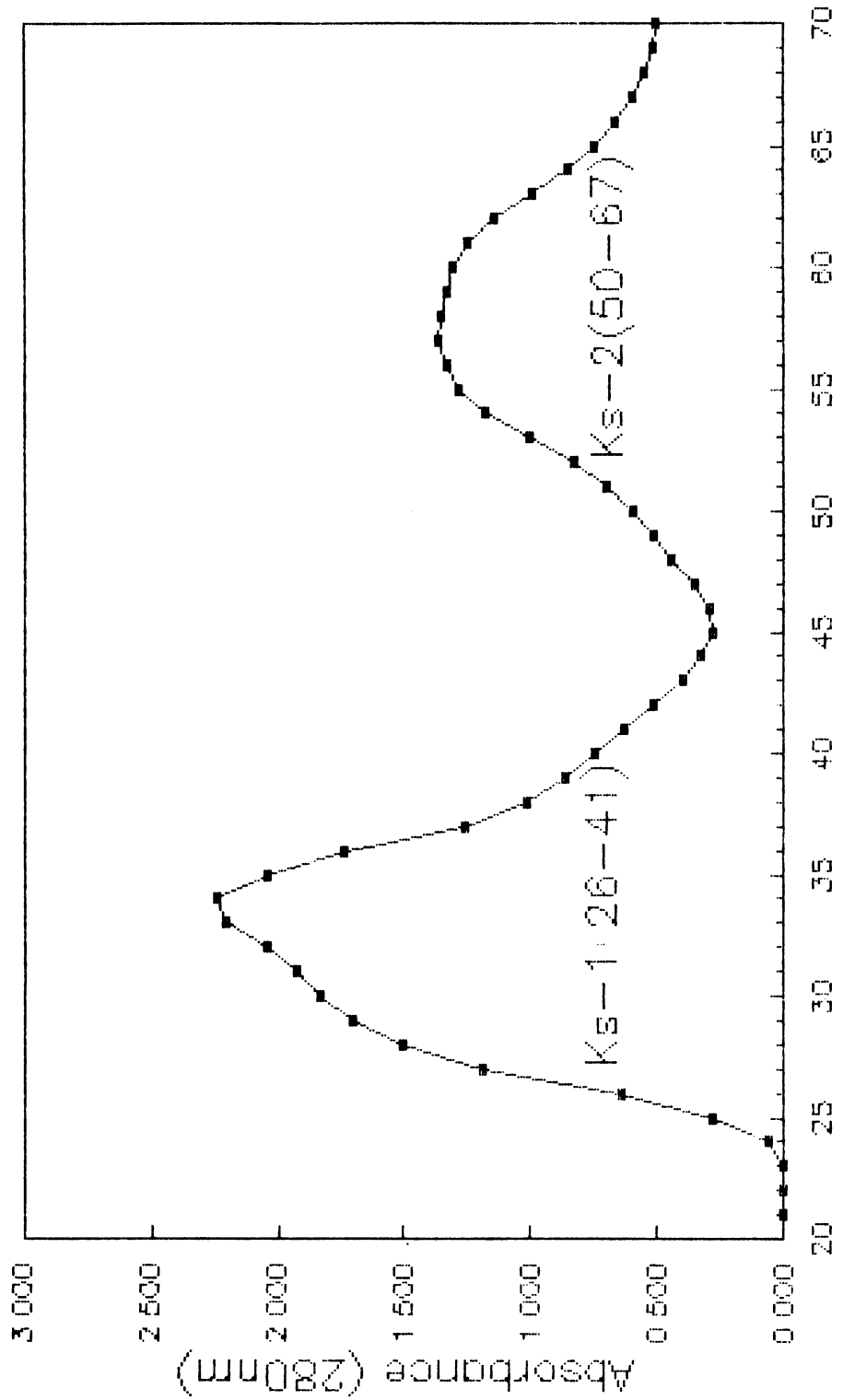


Figure 1. Ks-1 and Ks-2 fractions on a Sephacryl S-300 Column.

Figure 2. RP-HPLC of Ks-1 with 4.6mm x 250mm C-18 Vydac analytical column, 90 min. gradient, 0-70% B buffer. Flow rate was set at 1ml/min. with 2 min. fraction collections. Chart feed = min./cm, set at 2 min./cm or 0.5cm/min.

Figure 3. RP-HPLC of Ks-2 with 4.6mm x 250mm C-18 Vydac analytical column. 90 min. gradient, 0-70% B buffer. Other parameters same as Figure 2.

* Photo inserts represent 12.5% SDS-PAGE analysis of fractions solubilized in 1x SDS sample buffer at 100^{0}C for 3 min.

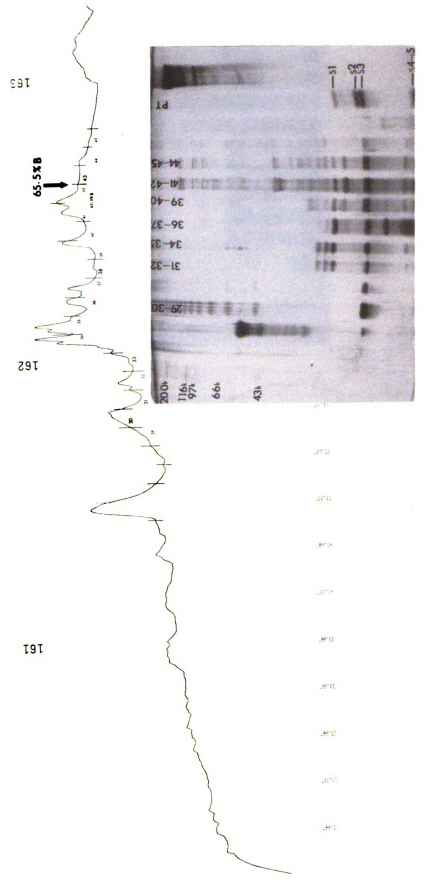

163
65.538
162
161
S1
S2
S3
PI
44-45
42-42
39-40
36-37
34-35
31-32
29-30
200
116
97
66
43

Figure 2

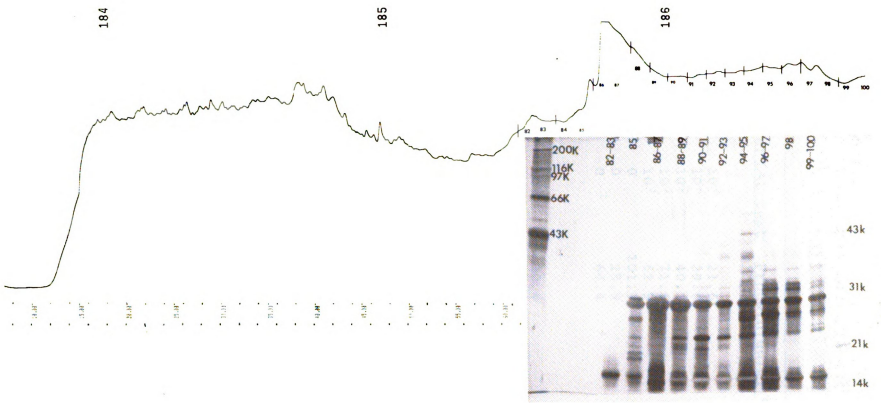

184
185
186
200K
116K
97K
66K
43K
82-83
85
86-87
88-89
90-91
92-93
94-95
96-97
98
99-100
43k
31k
21k
14k

Figure 3

Table 1. Protein and endotoxin concentrations in Ks-1 and Ks-2 fractions obtained from an analytical RP-HPLC column.

Fraction[a]	%B buffer[b]	LAL test[c]	Protein(ug/ml)[d]	EU/ug
Ks-1				
25-26	40.56	10^{-4}	21.6	115.7
29-30	46.80	10^{-4}	29.6	84.5
31-32	49.92	10^{-2}	40.1	0.623
36-37	57.72	10^{-2}	71.6	0.349
39-40	62.40	10^{-1}	62.9	0.039
41-42	65.52	0	101.3	0.0
44-45	70.20	0	25.9	0.0
49-50	78.00	0	64.4	0.0
Ks-2				
82-83	51.48	10^{-2}	30.4	0.82
84-85	54.60	0	43.6	0.0
88-89	60.84	0	92.3	0.0
90-91	63.96	0	82.0	0.0
92-93	67.08	0	61.7	0.0
94-95	70.20	0	83.2	0.0
96-97	73.32	0	90.4	0.0
99-100	78.00	0	101.9	0.0

a) 2-two minute fractions were combined to = 4ml/fraction.

b) B buffer consisted of acetonitrile:isopropyl alcohol: Milli-Q water in a ratio of 65:25:15.

c) Endotoxin determined by LAL gelation test. Last test dilution to show positive reaction.
   1- E. coli std. clots with 0.25EU/ml of U.S. std. endo.
   2- 0.25 EU/(dil.factor x ug/ml prot.) = EU/ug
   3- EU/ug x total prot. ug/ml = Total EU/ml

d) Protein determined by Beckman DU 64 spectrophotometer at 320nm, 280nm, and 260nm and calculated by the Warburg method.

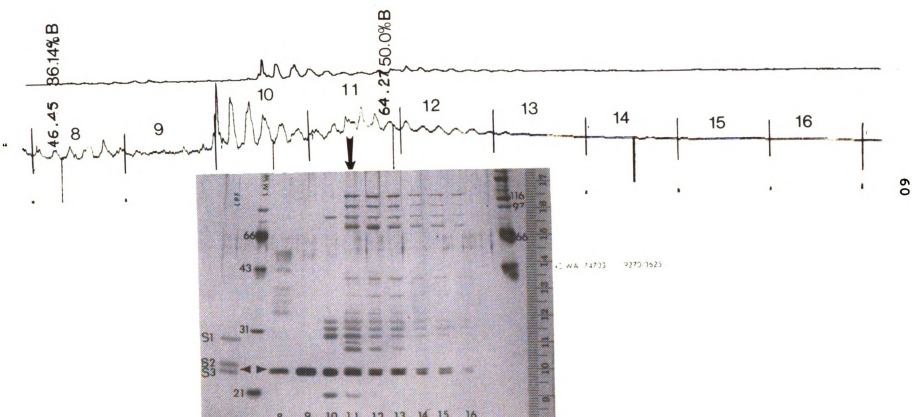
Abbreviations

EU = Endotoxin Units
LAL = Limulus Amoebocyte Lysate

Figure 4. RP-HPLC chromatogram of Ks-1 with preparative C-18 Vydac 22mm x 250mm co Flow rate was 30 ml/ min. with the Waters LC 3000 system and low pressure mixing. Fractions were collected over 5 min. interval = 150ml/fraction.

Figure 5. RP-HPLC chromatogram of Ks-2 with preparative C-18 Vydac 22mm x 250mm column. Flow rate of 30 ml.min. with the Waters LC 3000 system and low pressure mixing. Fractions were collected over 5 min. interval = 150ml/fraction.

* Photo inserts show 12.5% SDS-PAGE analysis of fractions solubilized in 1x SDS sample buffer at 100^{0}C for 3 min.


86.14%B
46.45
50.0%B
64.27
8
9
10
11
12
13
14
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16
S1
S2
S3
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116
97
8 9 10 11 12 13 14 15 16

Figure 4

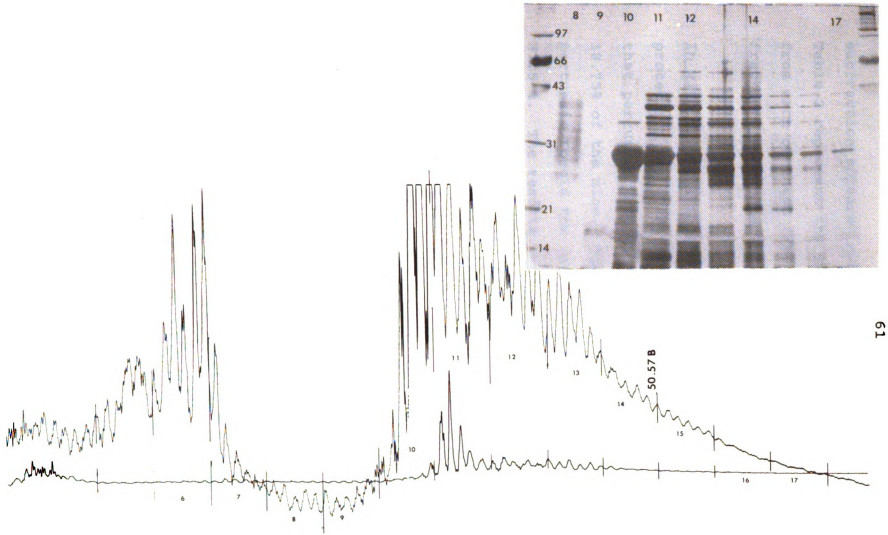

Figure 5

subfraction appeared around 30% B-buffer. The data in Table 2 represent the endotoxin and protein data obtained from each of these fractions. Based on these data, selected fractions were used for in vivo studies.

In vivo protection assays. The results of two mouse protection tests are shown in Table 3. The data indicated that pertussis toxoid alone only protected between 6.25 and 18.75% of the mice. When 2ug Ks-1 was added to 5ug pertussis toxoid the level of protection increased to 87.5 %. The results indicated that some component(s) in Ks-1 was enhancing the level of protection. When RP-HPLC isolated subfractions of Ks-1 were added either individually or pooled, the same level of protection was not observed. One fraction that appeared different from the others was F-11, which showed a difference of 18.75% as compared to 6.25% for F-9 and F-14. The pool which also contained F-11 did not confirm the results obtained with PTx and F-11 alone. Ks-1 subfraction alone demonstrated 43.7% protection which was a difference of 24.95 % over pertussis toxoid alone. The data with Ks-2 and its subfractions added to pertussis toxoid demonstrated some differences in Ks-2/F-14, F-17 and the pool. Ks-2 fractions individually did not improve protection.

The results of another experiment conducted in mice to determine the influence of FHA with pertussis toxoid, are shown in Table 4. The addition of FHA to 5ug of PTx showed

Table 2. Protein and endotoxin concentrations in Ks-1 and Ks-2 fractions resolved from a preparative RP-HPLC column.

Fraction	%B buffer[a]	Prot.(ug/ml)	EU/ug[b]
Ks-1			
F8	38.90	3.1	0.81
F9	42.79	15.0	0.17
F10	46.68	5.5	0.45
F11	50.57	10.9	0.23
F12	54.46	1.5	1.67
F13	58.35	1.0	2.50
F14	62.24	8.3	0.30
F15	66.13	3.4	0.73
F16	70.02	1.4	0.18
Ks-2			
F9	31.12	33.5	0.075
F10	35.01	476.9	0.005
F11	38.90	301.3	0.008
F12	42.79	182.2	0.014
F13	46.68	101.8	0.024
F14	50.57	72.9	0.034
F15	54.46	41.4	0.060
F16	58.35	26.1	0.096
F17	62.24	18.6	0.134

a) B buffer consisted of 0.1% TFA in acetonitrile.

b) Endotoxin units (EU) determined by LAL test same as in Table 1. Last test dilution showing a positive reaction was 1:10.

Table 3. Pertussis mouse protection test.

Fraction	Dose/0.5ml	Tot.EU	S/T	%Surv.	%Diff.
PTx	5ug	0.85	3/16	18.75	
PTx+Ks-1	5ug+2ug	68.85×10^5	14/16	87.50	68.75
PTx+F9	5ug+2ug	1.19	4/16	25.00	6.25
PTx+F10	5ug+2ug	1.75	2/16	12.50	0.00
PTx+F11	5ug+2ug	1.31	6/16	37.50	18.75
PTx+F14	5ug+2ug	1.45	4/16	25.00	6.25
PTx+Pool	5ug+8ug	3.15	4/16	25.00	6.25
Ks-1	2ug	68.00×10^5	7/16	43.70	24.95
F9	2ug	0.34	0/16	0.00	0.00
F10	2ug	0.90	1/16	6.25	0.00
F11	2ug	0.46	3/16	18.75	0.00
F14	2ug	0.60	2/16	12.50	0.00
Pool	8ug	2.30	3/16	18.75	0.00
PTx	5ug	0.85	1/16	6.25	
PTx+Ks-2	5ug+2ug	1344.94	3/16	18.75	12.50
PTx+F10	5ug+2ug	0.86	0/16	0.00	0.00
PTx+F12	5ug+2ug	0.88	3/16	18.75	12.50
PTx+F14	5ug+2ug	0.92	5/16	31.25	25.00
PTx+F17	5ug+2ug	1.12	4/16	25.00	18.75
PTx+Pool	5ug+8ug	1.22	4/16	25.00	18.75
Ks-2	2ug	1344.09	2/16	12.50	6.25
F10	2ug	0.01	0/16	0.00	0.00
F12	2ug	0.03	1/16	6.25	0.00
F14	2ug	0.07	1/16	6.25	0.00
F17	2ug	0.27	1/16	6.25	0.00
Pool	8ug	0.38	1/16	6.25	0.00

Controls: 1×10^{-4} opacity units/0.03ml = 0/18

Abbreviation: EU=Endotoxin units.
PTx=Pertussis toxoid (Lot 9=0.17EU/ug)
FHA=Filamentous hemagglutinin (Lot 3=0.7EU/ug)
S/T=Survivors/Total no.
%Surv.=% Survivors
%Diff.=% Difference

Table 4. Pertussis mouse protection test

Vaccine	1ug each PTx+FHA Tot.EU	S/T	%Surv.	%Diff	5ug each PTx+FHA Tot.EU	S/T	%Surv.	%Diff.
PTx+FHA	0.87	1/9	11.1		4.35	4/9	44.4	
PTx+FHA +2ug Ks-1	68.87*	5/7	71.4	60.30	72.35*	8/10	80.00	35.60
+2ug F9	1.21	2/8	25.0	13.90	4.69	6/9	66.70	22.30
+2ug F11	1.33	5/10	50.0	38.90	4.81	6/10	60.00	15.60
+2ug F14	1.47	2/10	20.0	8.90	4.95	5/10	50.00	5.60
+2ugKs-2	1344.96	3/9	33.30	22.20	1348.44	6/10	60.00	15.60
+2ug F10	0.88	3/9	33.30	22.20	4.36	2/10	20.00	0.00
+2ug F12	0.90	3/10	30.00	18.90	4.38	5/10	50.00	5.60
+2ug F14	0.94	4/9	44.40	33.30	4.42	3/9	33.30	0.00
+2ug F17	1.14	4/8	50.00	38.90	4.26	3/9	33.30	0.00

Controls: 1 x 10^{-4} opacity units/0.03ml = 0/10 survivors

Abbreviations
EU = Endotoxin Units
PTx = Pertussis toxoid lot 9 (0.17 EU/ug)
FHA = Filamentous hemagglutinin lot 3 (0.7 EU/ug)
S/T = Survivors/Total no.
%Surv. = %survivors

* = number shown x 10^5

an increase of 25.65% when compared with 5ug pertussis toxoid alone in Table 3. The addition of Ks-1 to PTx and FHA at the 1ug level demonstrated an increase of 60.3%. When the individual Ks-1 subfractions were added to PTx and FHA none of them were as good as Ks-1 alone. The subfraction F-11 did provide more protection when compared to F-9 or F-14. Two micrograms of Ks-2 fraction added to 1ug each of PTx and FHA did not provide the same protection as Ks-1. In this experiment the individual subfractions Ks-2 /F10 and F12 were not all that different from Ks-2 alone. Ks-2 fractions F-14 and F-17 did seem to indicate some improvement. The data at 5ug of PTx and FHA with the addition of Ks-1 showed an improvement, but the difference was not as noticeable as at the 1ug level of PTx and FHA. The subfractions of Ks-1 and Ks-2 were not as different when combined with 5ug each of PTx and FHA.

To further characterize certain peptide bands that had been seen in the original Ks-1 and Ks-2 RP-HPLC fractions, B. pertussis culture filtrate from sublot 60 was analyzed on SDS-PAGE (Figure 6). Bands that appeared similar to those in the original stock material were eluted from the gel, pooled according to respective molecular weights, concentrated in a speed-vac evaporator-concentrator and resuspended in the appropriate buffer. The individual peptides in concentrated form would be free of influence from other components found in RP-HPLC fractions. Eluted bands, assayed for contamination with pertussis toxin and

Figure 6. Sublot 60 B. pertussis peak "K" concentrated 70X, solubilized in 1x SDS sample buffer at 55°C for 5 minutes and loaded at 20ul per lane. Fragments cut from 7.5% and 10% gels for in vivo and in vitro experiments are indicated on the right side. High and low molecular weight standards are on the left.

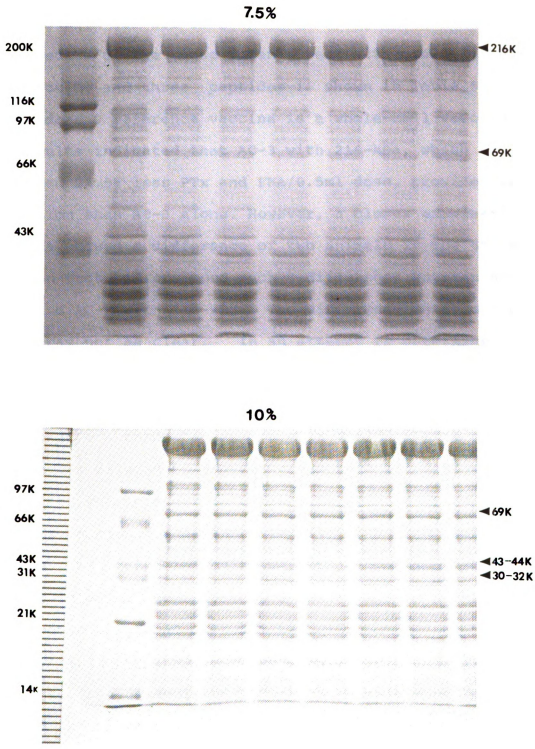
7.5%
200K
116K
97K
66K
43K
216K
69K
10%
97K
66K
43K
31K
21K
14K
69K
43~44K
30~32K

Figure 6

FHA by the ELISA test and for endotoxin by the LAL test, were negative for all three parameters.

Results of the mouse potency test with acellular DTP-AC-3 vaccine and three peptides is shown in Table 5. The US Standard 9 reference vaccine is a whole-cell vaccine. The results indicated that AC-3 with 216-kDa, which contained 23.5% less PTx and FHA/0.5ml dose, provided better protection than AC-3 alone. However, a closer examination of the data showed a difference of two animals in the third dilution that had produced such a difference between AC-3 alone and AC-3 with 216-kDa. The difference between the two groups was not as clear. In an attempt to determine the influence of the aluminum component plus endotoxin associated with diphtheria and tetanus toxoids, a second experiment was conducted as shown in Table 6. The AC-3 vaccine was diluted to provide 2.5ug each of pertussis toxoid and FHA in order to compare it with the same level of these two components unassociated with DTP-AC-3 acellular vaccine. The three molecular weight components eluted from SDS-PAGE did not show any real improvements in protection when combined with AC-3 or PTx plus FHA.

In vivo adjuvant studies. The question of a possible adjuvant effect associated with peak "K" components was studied in guinea pigs immunized with unadsorbed (1X) rhesus diploid rabies vaccine (RDRV), either alone or in combination with various fractions. A preliminary titration of 1X RDRV was done to determine a minimum dose that would

Table 5. Pertussis vaccine potency test showing effect of three subunits on protection activity of DTP-AC-3 vaccine.

Vaccine	%/0.5ml[a]	Amt.[b]	S/T	%Prot.	ED^{50}[c]	Units[d]
US Std.9	NA	0.04	12/16	75.0	.007	12.00
		0.008	10/16	62.5		
		0.0016	3/16	18.7		
AC-3[e]	100.00	0.08	13/16	81.2	.0107	7.89
	(25ugPTx	0.016	10/16	62.5		
	25ugFHA)	0.0032	4/16	25.0		
AC-3+216k	76.45	0.08	13/16	81.2	.0071	11.80
	(19.1ugPTx	0.016	10/16	62.5		
	19.1ugFHA	0.0032	6/16	37.5		
	7.6ug216k)					
AC-3+69k	77.16	0.08	13/16	81.2	.0138	6.10
	(19.3ugPTx	0.016	7/16	43.7		
	19.3ugFHA	0.0032	5/16	31.2		
	7.7ug69k)					
AC-3+44k	74.18	0.08	15/16	93.7	.0143	5.90
	(18.5ugPTx	0.016	7/16	43.7		
	18.5ugFHA	0.0032	3/16	18.7		
	7.4ug44k)					

a) The %component/0.5ml dose is the actual amount of each component after the addition of a volume containing 10ug of the peptide.

b) Amount injected/ml of original 0.5ml dose.

c) ED^{50} = amount of vaccine needed to protect half the injected mice (ml/mouse).

d) Units = units/total human dose (1.5ml)

e) Acellular DTP-AC-3 vaccine contained 50ug PTx, 50ugFHA, 30Lf diphtheria, 30Lf tetanus, 1.0mg Al and 0.01% Thimerosole/ml. The endotoxin level for Diphtheria and Tetanus toxoids is not monitored.

FHA = 0.7EU/ug x 50ug = 35EU (endotoxin units)
PTx = 0.17EU/ug x 50ug = 8.5EU
AC-3: 0.08 = 3.48 EU
0.016 = 0.695 EU
0.0032 = 0.139 EU

Table 6. Mouse protection test with acellular DPT-AC-3 vaccine[a] and peptide components.

Vaccine	Dose/0.5ml	s/t[b]	%Protected
AC-3	2.5ug each PTx+FHA	14/19	74.0
AC-3+216kDa	2.5ug each	12/20	60.0
AC-3+69kDa	2.5ug each	11/19	58.0
AC-3+44kD	2.5ug each	14/20	70.0
PTx+FHA	2.5ug each	6/19	32.0
PTx+FHA+216kda	2.5ug each	3/20	15.0
PTx+FHA+69kDa	2.5ug each	5/18	28.0
PTx+FHA+44kDa	2.5ug each	7/20	35.0

Unvac. controls: $1x10^{-4}$ o.u./0.03 ml = 1/10 survivors

a) AC-3 = DTP-AC-3 with same component concentration as shown in Table 5. The vaccine was diluted to give 2.5ug each of PTx and FHA.

FHA = 0.7 EU/ug x 2.5ug = 1.75 EU
PTx = 0.17EU/ug x 2.5ug = 0.425EU
Peptide = Negative for endotoxin
Each vaccine contains: 2.17 EU

b) s/t = Survivors/total (20 mice per group)

elicit an antibody response in 50% or less of the animals vaccinated. Antibody response to the vaccine was measured by the Rapid Fluorescent Focus Inhibition Test (RFFIT) at 14 and 21 days post vaccination. An example of a positive cell culture infection with rabies adapted virus CVS-11 is shown in Figure 7. The time of appearance of antibody and number of animals responding are presented in Tables 7 and 8. These two tables represent the combined results of two separate experiments. All fractions enhanced the antibody response by 14 days as compared to the rabies vaccine alone. RDRV with Ks-1/ F-9 (Table 7) demonstrated a greater increase at 14 days in the number responding over the controls. RDRV + Ks-1/F-11, the fraction that had indicated possible protection in mice, did not increase antibody level as well by 14 days. By 21 days there were no real differences between the groups. The data in Table 8 showed similar results with Ks-2 fractions at 14 days. All groups induced antibody over rabies vaccine alone. At 21 days post vaccination RDRV with F-14 indicated an increase in antibody level at 10ug and 1ug by difference of 22% and 16%, respectively. The level of endotoxin in Ks-1 and Ks-2 and their repective subfractions is the same as indicated in the mouse protection tests.

Cellular and humoral immune response. Rabbits (8-10) weeks old) were initially immunized with PTx and FHA alone or with the addition of Ks-1/F-11 or Ks-2/F-14, as shown in the

Figure 7. Fluorescein conjugated anti-rabies antibody staining of rabies CVS-11 infected BHK-21 cells.

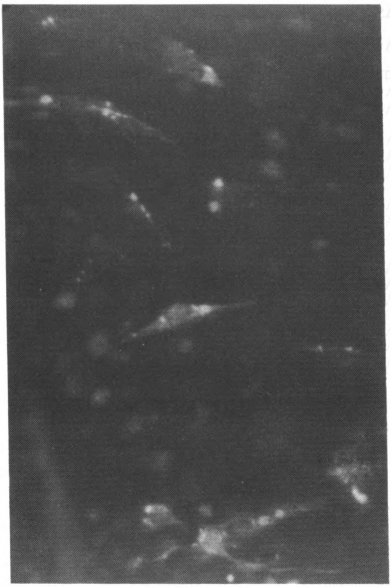

Figure 7

Table 7. Antibody response in guinea pigs immunized with (1x) Rhesus Diploid Rabies Vaccine (RDRV) and Ks-1 fractions.

Vaccine	Dose	14 Days PI P/T	14 Days PI %	21 Days PI P/T	21 Days PI %
RDRV	.025*	0/9	0	5/9	55
RDRV+Ks-1	.025+10ug	ND		ND	
	+ 1ug	0/5	0	0/5	0
	+ 0.1ug	2/5	40	2/5	40
RDRV+F9	.025+10ug	3/5	60	3/5	60
	+ 1ug	4/10	40	6/10	60
	+ 0.1ug	3/10	30	3/10	30
RDRV+F10	.025+10ug	0/5	0	0/5	0
	+ 1ug	1/5	20	3/4	75
	+ 0.1ug	1/5	20	3/5	60
RDRV+F11	.025+10ug	0/5	0	1/5	20
	+ 1ug	1/10	10	4/10	40
	+ 0.1ug	0/10	0	4/10	40
RDRV+F14	.025+10ug	1/5	20	1/4	25
	+ 1ug	1/10	10	5/10	50
	+ 0.1ug	2/10	20	4/10	40

* RDRV 1X Vaccine was diluted 1:4 and 0.1ml inoculated/animal. Dose .025 = 4988.15 pfu

Abbreviations:
PI = Post Immunization
P/T = Number positive /Total no. inoculated
ND = Not Done

Table 8. Antibody response in guinea pigs immunized with (1X) Rhesus Diploid Rabies Vaccine (RDRV) and Ks-2 fractions.

Vaccine	Dose	14 Days PI P/T	14 Days PI %	21 Days PI P/T	21 Days PI %
RDRV	.025*	0/9	0	5/9	55
RDRV+Ks-2	.025+10ug	0/5	0	0/5	0
	+ 1ug	0/5	0	2/5	40
	+ 0.1ug	2/5	40	3/5	60
RDRV+F10	.025+10ug	1/10	10	6/10	60
	+ 1ug	0/10	0	3/9	33
	+ 0.1ug	1/10	10	3/10	30
RDRV+F12	.025+10ug	1/10	10	5/10	50
	+ 1ug	2/10	20	5/10	50
	+ 0.1ug	1/10	10	2/10	20
RDRV+F14	.025+10ug	3/10	30	7/9	77
	+ 1ug	4/10	40	5/7	71
	+ 0.1ug	2/10	20	3/10	30
RDRV+F17	.025+10ug	1/10	10	5/9	55
	+ 1ug	1/10	10	3/10	30
	+ 0.1ug	2/10	20	2/10	20

*RDRV 1X vaccine was diluted 1:4 and inoculated 0.1ml per animal. Dose = 4988.15 pfu

Abbreviation:
PI = Post Immunizations
P/T = Number Positive /Total no.

chart, followed by a booster 3 months later with the same combination of vaccine components.

Group	Rabbit #.	LPF-tox.11	FHA-7	Ks-1/F11	Ks-2/F14
I	AI171/124	25ug	25ug		
II	AI107/116	25ug	25ug	10ug	
III	AI134/157	25ug	25ug		10ug
IV	AI199/174	0	0	0	0

Two milliliters of each vaccine with 0.25mg aluminum/ml was prepared. Each rabbit was given 2ml, 1ml per popliteal lymph node.

Experiments were designed to study the optimal conditions for cell-mediated immune response such as time of peripheral blood lymphocyte collection, number of lymphocytes needed per test condition, and the stimulation time and level of antigens and mitogens required. There was little or no evidence of tritiated thymidine (Sp. act. 2 Ci/mMol, 1 uCi/well) uptake by lymphocytes from either the initial immunization or the first booster. In wells were there was incorporation of tritium, the variation between counts per triplicate wells was greater than 2 standard deviations of the mean. The same was true with lymphocytes stimulated with PHA, Con-A or LPS from E. coli. The wash fluids contained high counts indicating that the cells were not incorporating the tritium into their DNA. In an attempt to resolve this problem, a second booster was given 10

months later with 69-kDa and 43-44-kDa peptides. Stimulation of PBL with PHA or the respective antigens at 14, 21, 28, and 35 days still did not produce the expected results. In two final experiments at 42 and 49 days, tritiated thymidine with a higher specific activity (Sp. act. 80 Ci/mMol) was used. There was an increase in cellular uptake in some wells; but, low counts and variation between triplicate wells were still factors that made the data difficult to interpret. Cells that had been cultured in 12 x 75 mm tubes and stimulated with antigen were monitored by ELISA after 10 days at 35^0C at 5% CO_2 for the presence of antibody. There was no evidence of antibody production in the fluids. To determine whether the rabbits had responded to all or part of the experimental vaccines their sera were tested for neutralizing antibody by the CHO cell assay and PT antibody by the ELISA technique (Table 9). The results indicate that the rabbits did respond to all or part of the antigens.

Preparation of additional RP-HPLC fraction stocks. Peak "K" eluate was obtained to prepare additional stocks of the RP-HPLC fractions. Pooled sublots were concentrated 20 times and applied to the sephacryl S-300 column as before. It appeared that current lots of peak "K" no longer produced the characteristic Ks-1 and Ks-2 peaks from the sephacryl S-300 column (Figure 8). Further analysis of this material on RP-HPLC with the preparative C-18 column and exclusion chromatography with the TSK-G-3000 column supported these observations. The method for pertussis toxin and FHA

Table 9. Antibody response in rabbits immunized with pertussis toxoid, filamentous hemagglutinin, and subcellular components.

Group	Vaccine	Rabbit ID#	14 Days PI 2nd Booster Neut.Ab.[a]	14 Days PI 2nd Booster ELISA Units[b]
I	PTx+FHA	171	10240	10.01
		124	2560	7.82
II	PTx+FHA[c]	107	10240	10.18
	(F-11)	116	5120	6.39
	69-kDa			
III	PTx+FHA[d]	134	5120	20.45
	(F-14)	157	2560	10.50
	44-kDA			
IV	None	199	<40	0.06
		174	<40	0.04

a) Neutralizing antibody determined by CHO cell assay. Data represent the reciprocal of the end point dilution.

b) Elisa Units calculated by parallel-line bioassay. The test serum and reference serum were plotted as $\log_{10}$ dilution versus optical density at 405nm. The units are determined by the anti-log difference between the x-intercepts of the reference and test curves redrawn with parallel slopes. Unitage of the test sample is determined by multiplying its relative titer by the assigned unitage of the reference serum, which was 100 for this set of data.

c) Rabbits received a primary immunization and booster with 25ug PTx + 25ug FHA and 10ug Ks-1/F11. The second booster contained 25ug each PTx and FHA + 10ug 69-kDa.

d) Rabbits received a primary immunization and booster with 25 ug each of PTx and FHA plus 10ug Ks-2/F14. The second booster contained 25ug each PTx and FHA plus 10ug of 44-kDA.

purification had changed which resulted in a difference in the peak "K" eluate. Several liters from sublot 55 were obtained and processed according to the original procedure. Sephacryl S-300 separation of peak "K" from sublot 55 resulted in the appearance of Ks-1 and Ks-2 peaks as seen previously. The RP-HPLC fractions generated from sublot 55 peaks Ks-1 and Ks-2 were used for the electroblotting, and immunostaining work.

SDS-PAGE Immunoelectroblotting. Immunostaining with monoclonal antibody BPE3 was used to identify the position of the 69-kDa peptide in sublot 55, Ks-1/F7 that had been electroblotted onto ProBlott. As shown in Figure 9, BPE3 faintly stained bands in the area of 69-kDa, as well as bands at approximately 100-98-kDa, 97-kDa, 45-kDa and 28-kDa. Several 7.5% gels were loaded with SL 55, Ks-1/F7, proteins were electroblotted onto ProBlott, stained with Coomassie and bands at 69-kDa were sliced from the membrane for direct sequencing in the Applied Biosystems 477A protein sequencer. There was no sequence obtained from these 69-kDa bands.

Several different monoclonal antibodies were used to characterize other bands in sublot 55 Ks-1/F-7. The monoclonals 9D4 (Figure 10A lane 4, specific for 216-kDa of Adenylate cyclase and its breakdown products), 8E7 (lane 5, specific for OMP 91kDa), P1E8 (lane 6, specific for 40kDa porin protein), BPE3 (lane 7, specific for 69-kDa) and BPF2 (lane 8, specific for Fimbriae 2) did not show specific

Figure 8. Sephacryl S-300 resolution of peak "K" sublot 53. Lane 1 contains crude peak "K". Lanes 2-6 represent the respective areas of protein in fractions eluted from the column. Protein concentration was measured at 280nm.

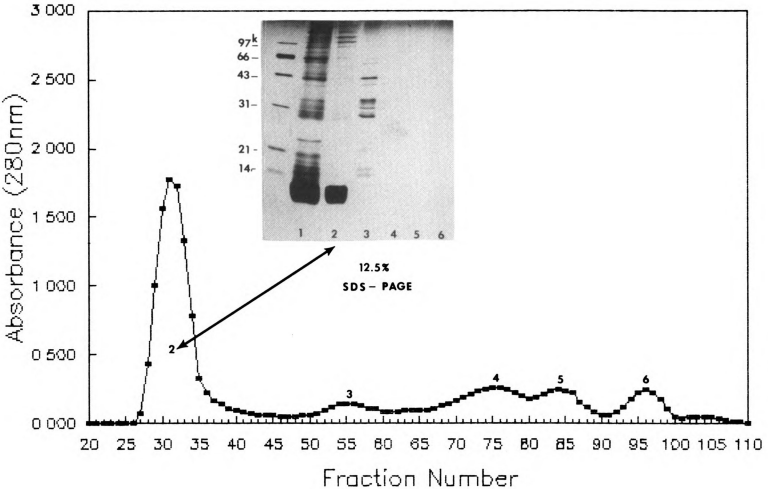

Figure 8 Sephacryl S-300 resolution of peak K sublot 53.

Figure 9. Monoclonal antibody BPE3, specific for 69-kDa outer membrane protein, used in Western immunoblotting technique to identify the 69-kDa peptide from sublot 55, Ks-1/RP-HPLC fraction 7. The band indicated with an arrowhead was the 69-kDa peptide selected for sequence analysis.

Lane 1 = High molecular weight standards.

Lane 2 = Sublot 55, Ks-1/F7 stained with Coomassie Blue R-250.

Lane 3 = Anti-mouse IgG peroxidase

Lane 4 = Anti-mouse IgM peroxidase

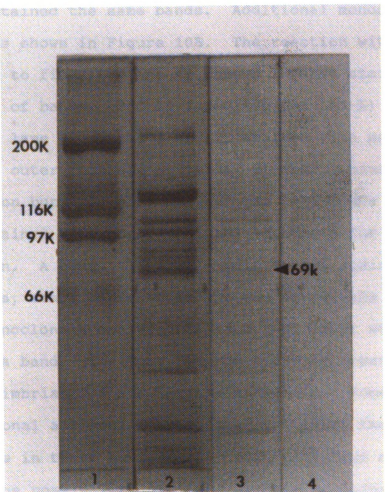

Figure 9

staining. A band between 97-116-kDa molecular weight markers, 98-100-kDa, was common to all monoclonals. BPE3 (lane 7, anti 69-kDa) and BPF2 (lane 8, anti fimbriae 2) also stained the same bands. Additional monoclonals were used as shown in Figure 10B. The reaction with monoclonal BPC 10 to fim 3/6 (lane 4) showed intense staining with a number of bands. BPG 10 (specific for LPS-A) stained the entire lane 5. Lanes 6 and 7, stained with monoclonals for 91-kDa outer membrane proteins, stained a band at that position but they also stained the 98-100-kDa band. Lane 8 was stained with monoclonal 1D7, specific for a 40-kDa porin protein. A band at approximately 45-kDa stained faintly, as well as, other bands including the 98-100-kDa band. All of the monoclonals analyzed in this experiment stained the 98-100-kDa band. The most intense staining occurred with the anti-fimbriae 3/6 and LPS-A monoclonals. None of the other monoclonal antibodies specifically stained their designated peptide in these Ks-1 RP-HPLC fractions from sublot 55.

The possibility the purification procedures had altered peptides or exposed different epitopes was examined. The RP-HPLC analysis of Ks-1/F7 from sublot 55 and the 216-kDa peptide eluted from the SDS-PAGE of crude peak "K" sublot 60 were immunostained with monoclonal antibody 9D4 (specific for 216-kDa peptide and its breakdown products) and with FHA specific P12H3 (Figure 11A). The focus on 216-kDa obtained by two different procedures (Lanes 2 and 7) and stained with 9D4 showed only faint staining of bands at 216-kDa and

Figure 10. Western blot-immunostaining of SL 55 Ks-1/F7 on 7.5% SDS-PAGE.

A: Lane 1. Pre-stained high MW stds.
Lane 2. High MW stds.
Lane 3. SL 55 Ks-1/F7 - Coomassie stain
Lane 4. " - 9D4 (216kda)
Lane 5. " - P8E7 (91kDa)
Lane 6. " - P1E8 (40kDa)
Lane 7. " - BPE3 (69kDa)
Lane 8. " - BPF2 (Fim.2)

B: Lane 1. Pre-stained HMW stds.
Lane 2. HMW stds.
Lane 3. SL 55 Ks-1/F7 - Coomassie
Lane 4. " - BPC 10 (Fim 3/6)
Lane 5. " - BPG 10 (LPS-A)
Lane 6. " - P2F1 (91kDa)
Lane 7. " - 1F11 (91kDa)
Lane 8. " - 1D7 (40kDa porin)

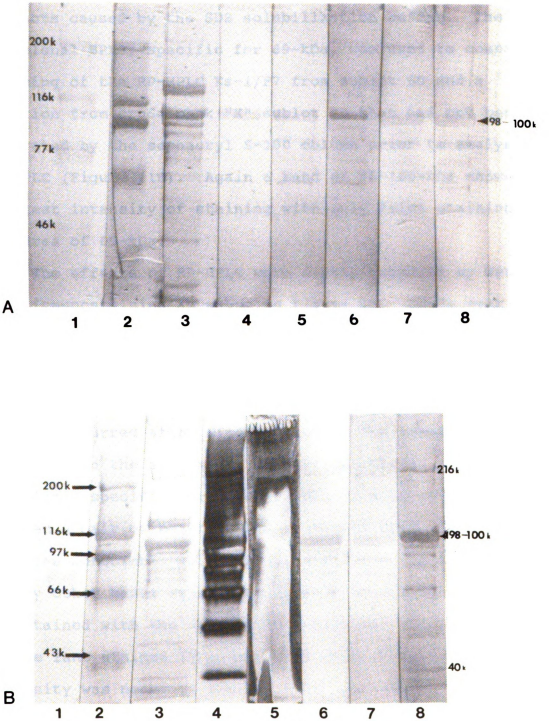
200k
116k
77k
46k
498 - 100k
A
1 2 3 4 5 6 7 8
200k
116k
97k
66k
43k
216k
498-100k
40k
B
1 2 3 4 5 6 7 8

Figure 10

98-100-kDa. Anti-FHA monoclonal P12H3 (Lanes 3 and 8) showedintense staining of the same bands at 216-kDa and 98-100-kDa, as well as additional bands, possibly breakdown products caused by the SDS solubilization buffer. The monoclonal BPE3, specific for 69-kDa, was used to compare staining of the RP-HPLC Ks-1/F7 from sublot 55 and a fraction from crude peak "K" sublot 60 that had not been separated by the sephacryl S-300 column prior to analysis on RP-HPLC (Figure 11B). Again a band at 98-100-kDa showed the greatest intensity of staining with only faint staining in the area of 69-kDa.

The effects of RP-HPLC were further studied by Western blot immunostaining as shown in Figure 12. Crude peak "K" (sublot 60) was analyzed by SDS-PAGE and Western blot (Figure 12A). The immunostaining with 9D4 stained the 216-kDa band with greater intensity than was seen previously (fading occurred after drying). Anti- FHA monoclonal P12H3 also stained the same bands as well as others. The monoclonal specific for 69-kDa (BPE3 in Lane 5) specifically stained a pair of bands in the 67-69-kDa area. The anti-fimbriae monoclonal antisera did not stain the 69-kDa band or any other bands except the band at 98-100-kDa. Lane 8 was stained with the anti-LPS-A monoclonal and although most of the lane stained intensely, the area of greatest intensity was noted in the low molecular weight peptide region (appears as a light area in the photograph). All three lanes in Figure 12B, RP-HPLC sublot 55 Ks-1/F7 (Lane

Figure 11A. Western blot-immunostaining with monoclonal antibodies.

7.5% SDS-PAGE: Solubilization @ 55^{0}C for 5 minutes.

Lane 1 = SL55, Ks-1/F7 (20ul) + Coomassie

Lane 2 = " + 9D4 (anti-216-kDa)

Lane 3 = " + P12H3 (anti-FHA)

Lane 4 = HMW stds. (20ul)

Lane 5 = HMW stds. (10ul)

Lane 6 = 216-kDa eluted from SL60 +Coomassie

Lane 7 = 216-kDa " " " + 9D4

Lane 8 = 216-kDa " " " + P12H3

Figure 11B. Western blot-immunostaining with monoclonal antibodies.

Lane 1 = HMW std.

Lane 2 = SL55, Ks-1/F7 (RP-HPLC) + Coomassie

Lane 3 = SL55, Ks-1/F7 (RP-HPLC) + BPE3 (69k)

Lane 4 = SL60 crude from RP-HPLC + Coomassie

Lane 5 = SL60 " " " + BPE3 (69k)

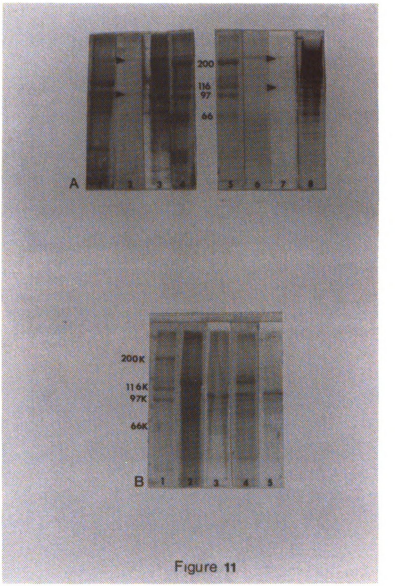

Figure 11

Figure 12A. Western blot immunostaining with monoclonal antibodies. Lanes contain crude SL60 concentrated 70X, 10ul per lane.

Lane 1 = HMW pre-stained stds.
Lane 2 = SL60 + Coomassie Blue R-250
Lane 3 = " + 9D4 (anti-216-kDa)
Lane 4 = " + P12H3 (anti-FHA)
Lane 5 = " + BPE3 (anti-69-kDa)
Lane 6 = " + BPF2 (anti-Fimbriae 2)
Lane 7 = " + BPC10 (anti-Fimbriae 3/6)
Lane 8 = " + BPG10 (anti-LPS-A)

Figure 12B. Western blot immunostaining with monoclonal antibody BPE3 specific for 69-kDa.

Lane 1 = HMW pre-stained stds.
Lane 2 = HMW stds.
Lane 3 = SL55, Ks-1/F7 (30ul)
Lane 4 = 69-kDa eluted from SL60 (30ul)
Lane 5 = 70X concentrated SL60 (10ul)

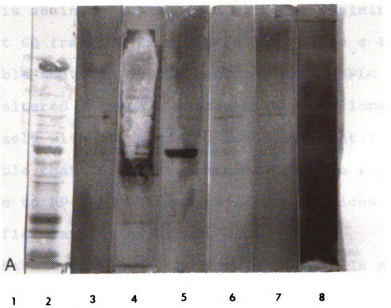

Figure 12

3), 69-kDa eluted from peak "K" sublot 60 (Lane 4) and crude sublot 60 (Lane 5), were stained with BPE3 (anti-69-kDA). If this staining is compared with the staining of RP-HPLC sublot 60 fraction from Figure 11B (Lanes 4 & 5), it is possible that the 69-kDa band in the RP-HPLC preparation has been altered in such a manner that it no longer stains intensely with its specific monoclonal antibody. It is also possible that the faint staining noted in Figures 9 and 10 is due to RP-HPLC alteration of the peptides such that no specific staining occurred.

Rabbit polyclonal Reference Bordetella pertussis Factor 1,2,3 and 6 antisera were used to stain electroblotted peptides from peak "K" sublot 60. Strain 18334 of B. pertussis used in the work is of serotype 1 through 6. In lanes 4 and 5 of Figure 13 there are several peptide band that stained more intensely than others; however, the 69-kDa band stained with serotype 1 and 2 agglutinogens more than with types 3 and 6.

Figure 13. Reference B. pertussis Factor polyclonal antisera staining of 70X concentrated crude sublot 60. 10% SDS-PAGE.

Ref. Factor antisera diluted 1:300 from Div. Biological Standards, National Inst. of Health, Bethesda, Md., 20014.

Lane 1 = Pre-stained LMW stds.
Lane 2 = LMW stds.
Lane 3 = SL60 + Coomassie
Lane 4 = " + Agglutinogen Ref. Factor 1
Lane 5 = " + " " " 2
Lane 6 = " + " " " 3
Lane 7 = " + " " " 6

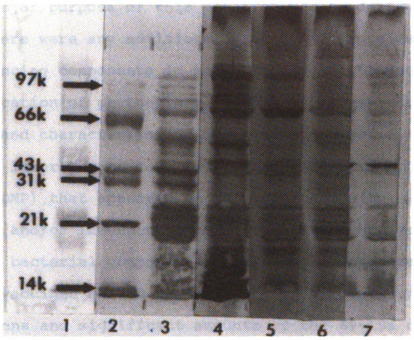

Figure 13

DISCUSSION

The major purpose of this research was to determine whether there were any additional protective proteins or other enhancing components in peak "K" eluate obtained from the purification of pertussis toxin and to attempt the isolation and characterization of these proteins.

Other laboratories have reported on outer membrane proteins (OMP) that appeared to be associated with protection and/or immunoenhancing activities. Analysis of additional bacterial components found in the Japanese acellular vaccines showed the presence of trace amounts of agglutinogens and significant amounts of the 69-kDa outer membrane protein (Shahin et al., 1990). Interest in the 69-kDa OMP as a potential vaccine component was initiated by the observations that a 68-kDa OMP of B. bronchiceptica conferred protective immunity in susceptible animals (Novotny et al., 1985a,b), its identification on the surface of virulent B. pertussis strains, and the discovery of a strong immune response to the 69-kDa protein of B. pertussis from the sera of whole-cell vaccine recipients or convalescents (Shahin et al., 1990; Thomas et al., 1989). Bacterial fimbriae proteins, Fim 2 and Fim 3 (MW 22.5k and 22k respectively, Robinson et al., 1989), formerly

agglutinogens 2 and 3/6, were shown to be good immunogens and a mixture of these components have been incorporated into an acellular vaccine (Miller et al., 1990). Although no cellular role in the pathogenesis of pertussis has yet been ascribed to fimbriae, antibodies to fimbriae, FHA and PT inhibit the adhesion of B. pertussis to Vero cells and at low doses induce antibody in mice that protect against respiratory infection (Robinson et al., 1990). Adenylate cyclase, a 216-kDa holotoxin and its associated 70-kDa and 43-kDa proteins, along with pertussis toxin appear to be critical for the bacteria to cause a lethal infection. The incorporation of the adenylate cyclase protein into an acellular vaccine has also been considered (Weiss et al., 1986). A 30-kDa outer membrane protein was shown by Monji et al., (1986) to have strong adjuvant properties with purified Haemophilus influenzae type b capsular polysaccharide, polyribosyl ribitol phosphate (PRP). It has recently been reported that the OMP of gram negative bacteria contain 40-kDa porin proteins which form diffusion pores that separate molecules on the basis of size and charge. Because purified porin from an avirulent strain elicits an immune response in mice that was not protective, the purified porin from a virulent strain is being investigated for protective efficacy against respiratory challenge with virulent B. pertussis (Hannah et al., 1990). In light of these published reports on outer membrane proteins as possible candidates for inclusion in

acellular vaccines, there was a renewed interest in the finding by researchers at the Michigan Dept. of Public Health that two subfractions of peak "K", Ks-1 and Ks-2, contained some component(s) that improved the protective activity of purified pertussis toxoid (Shih _et al._, 1986).

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were used in this research as a rapid method of analyzing the protein content of peaks Ks-1 and Ks-2. Use of a trimethylsilylated silica gel (TSK-TMS-250) RP-HPLC column was described by Yamakawa _et al._, (1990) to isolate the subunit proteins of pertussis toxin. They demonstrated that RP-HPLC could be used as an analytical method to separate the subunits with high yield. They also demonstrated that the activity of the complex holotoxin could be reconstituted such that the LPF activity was comparable to native PT. Because of the significant resolving power of RP-HPLC the method was selected as a means of separating proteins for analysis by SDS-PAGE, for _in vivo_ studies of protection and immunoenhancing activities and for purification of peptides for sequencing analysis.

The activity of several RP-HPLC fractions was assayed by the mouse protection test. The data presented in Table 3 through 6 indicated that activity was associated with the presence of endotoxin. This was especially noticeable in Tables 5 and 6 where purified bands negative for PT, FHA and endotoxin did not improve protection over the use of the

acellular vaccine alone. Reduction of endotoxin resulted in reduced protection. This is not surprising since the enhancing activity of endotoxin has previously been demonstrated. The ability of endotoxin to induce polyclonal B-lymphocyte activation has been shown (Haeffner-Cavaillon et al.,1982). Evidence supporting mitogenic and adjuvant activity of pertussis toxin and lipopolysaccharide (LPS) endotoxin has also been reported (Fish et al., 1984: Nogimori et al., 1984; 1986; Kolb et al., 1990). The potent adjuvant activity of B. pertussis cells (Munoz, 1963) is due to the endotoxin (Farthing, 1961) and in pertussis vaccines the adjuvant may enhance the efficacy of protective antigens.

Even with the endotoxin present in AC-3, PTx and FHA, it is a possibility that the amount of the individual purified peptide was not sufficient to be able to detect an influence due to the protein alone (Tables 6). It may have been possible that a combination of the peptides was needed to form a configuration that would have effected a noticeable increase in the level of protection. The data in Tables 3 and 4 with fractions containing many peptides seem to support this hypothesis. Ks-1 with pertussis toxin certainly had a high level of endotoxin. The results of PTx with each of the Ks-1 subfractions showed that the concentration of endotoxin was low in each group and yet one of these, Ks-1/F11, did show improvement as compared to the other three. There is a similar situation in the Ks-2 group

with Ks-2/F14 and F17. The data in Table 4 indicate this same trend at the 1ug level of PTx+FHA. Capiau et al., (1990) investigated the possible synergy between the 69-kDa OMP and other acellular vaccine components in IC challenge of mice. Their results indicated that 8ug of the 69-kDa component alone failed to confer any protection. A combination of 8ug PT + 25ug FHA gave an intermediate protection which was inferior to the whole cell vaccine control. A three-component combination of 8ug 69-kDa, 8ug PT and 25ug FHA gave an improved protection level similar to the control. This improvement over the two-component combination of PT-FHA was apparent only at the high dose and not at lower dilutions. In another test where successive dilutions of the 69-kDa OMP were combined with 2.5ug each of PT and FHA, a similar result was obtained. Possibly the data in Table 5 with AC-3 in combination with 216-kDa might be significant in view of this report.

The possibility of a separate protein or protein complex that had adjuvant properties was investigated in this work. A preliminary titration of 1X rabies vaccine was done to determine a level at which a minimal antibody response could be measured in 50% or less of animals vaccinated. The experimental design using minimum level in combination with some component in one of the fractions was done to show an adjuvant effect measured by increased time to onset of antibody induction, as well as increased number responding as compared to the controls. The data in Tables 7

and 8 indicated that there was some component in the RP-HPLC fractions that enhanced antibody production. The level of endotoxin associated with each group was the same as that in the protection tests. The results obtained with RDRV + Ks-1/F9 might possibly be due to the presence of pertussis toxin. Even though peak "K" was the result of pertussis toxin purification there was probably a residual amount left in the eluate. The SDS-PAGE of F9 is shown in Figure 4 and the only band present was a doublet at the same molecular weight associated with the S2 and S3 subunits of pertussis toxin. This fraction was not checked for PT activity by ELISA or histamine sensitizing activity. Fraction 14 from Ks-2 also appeared to express an enhancing activity. The SDS-PAGE of this fraction is shown in Figure 5. There are several bands in this fraction, but the one of interest is a band at
the 30-kDa level that might be similar to the 30-kDa peptide implicated to have adjuvant activity (Monji et al., 1986).

The rabbit was chosen for cell mediated immune response studies, because the respiratory infection in rabbits resembled that in humans (Carter and Preston, 1981); however, the data obtained did not support this choice. It was hoped that lymphocyte proliferation would prove to be a sensitive means of determining the interaction between individual peptides and the cells of the immune system. Haeffner-Cavaillon et al., (1982) used rabbit splenocytes to demonstrate the macrophage-dependent polyclonal

activation of B lymphocytes into immunoglobulin secreting cells by B. pertussis endotoxin. It was also pointed out that endotoxins do not stimulate rabbit peripheral lymphocytes; however, it was hoped that other antigens associated with the fractions or individual peptides would produce in vitro B-cell response and T-cell blast transformation as was shown by Wiertz et al., (1989) with human peripheral blood lymphocytes from convalescents. Broekhuyse and Van Vugt, (1989) and Sell et al., (1972) used rabbit peripheral blood lymphocytes for in vitro cellular immune responses. An improved method for lymphocyte isolation resulting in consistent recoveries of 65-75% PBL's and their use in lymphocyte transformation tests was described (Broekhuyse and Van Vugt, 1989). A possible lack of response with the RP-HPLC fractions may have been due to protein denaturation by organic solvents used to resolve proteins (Yamakawa et al., 1990). However, the lack of response to pertussis toxoid, FHA and mitogens is difficult to explain. Rabbit sera were tested in an ELISA for antibody to B. bronchiseptica by Dr. David Bemis, University of Tenn., Knoxville, Tenn. The data could not be quantitated for B. bronchiseptica alone because of the dual infection with B. pertussis. The results showed that all eight rabbits responded at a level higher than specific hyperimmune control sera. Pasteurellosis has been shown to affect the rabbit's immune response and limit the life of the animal(Dunbar and Schwoebel, 1990). To determine

whether the humoral immune system had been affected by this dual infection, the 8 rabbit sera were analyzed for PT neutralizing antibody by the CHO cell assay and anti-pertussis antibody by the ELISA test. Since the rabbits from the three inoculated groups were positive for PT antibody by these two tests it was concluded that their humoral immune system was still functional. The possibility that isolation of peripheral blood lymphocytes was not done exactly as described could have been a source of the lack of lymphocyte stimulation.

SDS-Page and Western blotting techniques used in this work served to point out some advantages and disadvantages with the system. The advantages involve ease of handling and fairly rapid identification of specific peptides with monoclonal antibodies. It also avoids problems with handling and storage of radioactive isotopes associated with radioimmunoassays and autoradiography. There are also disadvantages in that some substrates are carcinogenic, color fading can occur due to light and drying and non-specific binding of enzyme-conjugated antibodes has been observed in some situations (Tovey et al., 1987).

Because of the high acidity and the organic solvents used to elute proteins from the hydrophobic RP-HPLC column, protein denaturation possibly altered the structure of the proteins such that monoclonal antibodies against specific peptides did not recognize the altered epitopes and gave the appearance of non-specific staining. A comparison of the

methods of protein purification indicated that peptides obtained from crude peak "k" sublot 60 bands cut from SDS-gels and electroblotted were not influenced by the conditions of RP-HPLC and appeared to stain specifically with their respective monoclonal antibodies. One problem not resolved was the faint staining of 216-kDa with the adenylate cyclase specific monoclonal 9D4 and P12H3 anti-FHA monoclonal antibody. The faint staining may have been due to the low level of high molecular weight protein transfer characteristic of electroblotting. The heavy staining due to anti-FHA may indicate that the 216-kDa really is FHA or that both the 216-kDa and 220-kDa proteins of FHA were removed from the SDS-gel together and there is cross reaction occurring. The FHA staining is confusing in view of the fact that the ELISA technique indicated that the 216-kDa preparation from the gel was negative for FHA. This issue was not resolved.

The 69-kDa band for sequence analysis was identified by BPE3 specific monoclonal antibody from the RP-HPLC sublot 55 Ks-1/F7. The apparent non-specific staining that occurred may have resulted in the selection of the wrong peptide for sequencing. However, the fact that there was no sequence obtained may have been due to interference by Coomassie staining, residual buffer containing tris, glycine or SDS, lack of sufficient peptide for sequencing or blocking of the N-terminus due to a poor grade of chemical used in the process.

Specific staining with several monoclonal antibodies is shown in Figure 12. The staining of the 69-kDa band with BPE3 tends to support the existence of the peptide in the crude peak "k" material. The lack of fimbriae staining with BPF2 (anti- fimbriae 2) and BPC10 (anti-fimbriae 3/6) is in agreement with Brennan et al. (1988). The staining of the entire lane with the anti-LPS monoclonal BPG10 may be indicative of the endotoxin level in this preparation. The staining reactions with the Reference Factor polyclonal antisera indicated that 69-kDa peptide stained more intense with Reference Factors 1 and 2. Staining with Factor 3 was not as intense.

The importance of this research has been the characterization of an endotoxin component in peak "K" fractions Ks-1 and Ks-2. Peak Ks-1/F11 also appears to contain the 69-kDa protein, although the reduced biological activity in the RP-HPLC fraction was probably due to low levels of the components and some protein denaturation from the chaotropic solvents. The lack of activity in the peptide sliced from SDS-Page was probably due to loss of endotoxin and the low amount of the component. SDS-Page and Western blot techniques with specific monoclonal antibody identified this protein to be similar to that described by Brennan et al., (1988). The identification of a 216-kDa band was not clearly resolved and needs further study. Adjuvant properties in the Ks-1 and Ks-2 fractions seemed to be associated not only with endotoxin but possibly

contamination with pertussis toxin in the case of Ks-1/F11. The Ks-2/F14 is lower in endotoxin but it has a 30-kDa peptide which may be similar to the one described by Monji et al.,(1986). Its isolation by the methods described will help to characterize this peptide. The identification of the 44-kDa doublet was not done. The possibility that it was a break-down product of the 216-kDa adenylate cyclase protein was not confirmed by staining with monoclonal 9D4 in crude sublot 60 peak "K" preparations.

The high resolution of peptides obtained with RP-HPLC did prove to be the method of choice for structural analysis of peaks Ks-1 and Ks-2. The use of such fractions in vivo however did not provide a good means of determining the associated biological activity due to the protein denaturation which seemed to occur.

An attempt was made to provide another means of assaying protective and adjuvant activity by cell-mediated immune function. Future research toward this end may be aimed at using T-cell and B-cell hybridomas to study the adjuvant and mitogenic properties of individual peptides. The current mouse potency assay used to evaluate whole-cell pertussis vaccines is becoming less acceptable as a means of measuring the efficacy of acellular vaccine components.

SUMMARY

This research concerns the isolation and characterization of protective proteins or other subcellular components in an eluate obtained during the purification pertussis toxoid. Reverse Phase-High Performance Liquid Chromatography was used to isolate subfractions containing reduced levels of endotoxin that appeared to demonstrate protective activity in mice challenged with live pertussis organisms. Selected peptides within certain subfractions were further purified to the point that the endotoxin level was not evident. The reduction of endotoxin also resulted in the reduction of protective and immuno-enhancing activity. Western blot and immuno-staining techniques with monoclonal antibodies indicated some identity with peptides located in the outer membrane with those reported by other investigators. One major protein that has been identified as a candidate for acellular vaccines appeared to be in the preparations under investigation. The data obtained with the 69-kDa peptide in this research demonstrated similar characteristics to the 69-kDa outer membrane protein described by others. Several other peptides were tentatively identified but further research is needed.

RECOMMENDATIONS

Further investigation into combining isolated peptides to determine whether more than one peptide was needed to produce the desired effect, as well as dose levels would help to substantiate whether peptides could function without the close association of endotoxin. If indeed endotoxin is necessary, a method to analyze the effects of protein in that configuration would be needed. An experiment using isolated peptides, a peptide such as BSA that is completely unrelated and a media control group could be set up with measured increments of endotoxin added to each group to determine the amount needed to restore protective and/or adjuvant activity. Also, the threshold level at which endotoxin would be beneficial and not toxic to the host would be of interest.

An <u>in vitro</u> method to measure stimulation activity, such as human or mouse lymphocyte (B/T) hybridoma cell lines, might prove to be a means of replacing the mouse test. Stimulation of B cells to produce IgG into the media that could be assayed by ELISA would also reduce the need for radio-active isotope work used with lymphoblast transformation assays. A pool of lymphocytes obtained from syngeneic mice immunized with pertussis components and

stimulated with the same components *in vitro* could be used to sort out the cellular immune response associated with pertussis by labeling the cells with commercial preparations of monoclonal antibodies to the various subsets of B and T cells.

Additional work into the routes of inoculation of the various peptide components is also needed. The use of isolation facilities for animal studies would greatly improve the ability to perform cell-mediated immunological research.

Further analysis of the peptides eluting from the RP-HPLC column in the area of 50.57% B buffer would provide additional characterization of the proteins associated with the apparent biological activity.

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