HOST-PARASITE INTERACTIONS BETWEEN *BORDETELLA PERTUSSIS* AND HUMAN POLYMORPHONUCLEAR LEUKOCYTES

by

Lisa Lovett Steed

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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Host-parasite interactions between *Bordetella pertussis* and human polymorphonuclear leukocytes

Steed, Lisa Lovett, Ph.D.

The University of Arizona, 1991

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GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Lisa L. Steed

entitled Host-Parasite Interactions Between *Bordetella pertussis* and Human Polymorphonuclear Leukocytes

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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J. Glenn Songer — Date September 24, 1991

John N. Galgiani — Date September 24, 1991

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director — Date September 24, 1991

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ABSTRACT

Little is known regarding the interaction of *Bordetella pertussis* with polymorphonuclear leukocytes (PMNL) or the role PMNL play as an initial line of defense against *B. pertussis* infection. An *in vitro* system was developed to establish optimum conditions for the study of phagocytosis and killing of three virulent strains of *B. pertussis* and a series of derivative mutants using strictly human PMNL and sera. Optimum phagocytosis of *B. pertussis* occurred by opsonization with human anti-*B. pertussis* antibody (HAPA), while autologous normal sera (NS) did not induce significant phagocytosis. An antiserum made in rabbits against an avirulent strain was required as an opsonin for significant phagocytosis of several avirulent strains. Over 50% of all *B. pertussis* strains and mutants tested survived PMNL bactericidal activities while *Escherichia coli* controls were readily killed. Therefore, if internalized, *B. pertussis* has an innate ability to survive within human PMNL. No one virulence factor apppears to be superior to another in determining survivability. Electron microscopic studies using acid phosphatase as a lysosomal marker demonstrated that virulent *B. pertussis* strains are capable of surviving intracellularly within PMNL phagosomes and that such survival is due to inhibition of phagosome-lysosome fusion. PMNL respiratory burst activity is unaffected by internalized *B. pertussis*. This strongly suggests that inhibition of phagosome-lysosome fusion is the key mechanism of *B. pertussis* intracellular survival within PMNL.

CHAPTER I

INTRODUCTION

Epidemiology of Pertussis.

Pertussis, or whooping cough, is a highly contagious disease of the tracheobronchial tree caused by *Bordetella pertussis*. A strict human pathogen, *B. pertussis* exclusively adheres to and multiplies in association with ciliated epithelium (Olson, 1975; Manclark, 1984; Weiss, 1986). Although the organisms are not isolated outside the respiratory tract, a battery of virulence factors are generated which have local as well as systemic effects resulting in the symptoms characteristic of pertussis (Olson, 1975; Manclark, 1984; Weiss, 1986; Friedman, 1988). First described in the literature as an epidemic in Paris in 1578, pertussis had become endemic throughout Europe by the 17th century (Olson, 1975). As recently as 1948, pertussis was a leading cause of death in children under 14 years of age in the United States (Olson, 1975). Before the development of a whole cell *B. pertussis* vaccine, out of 115,000 to 270,000 pertussis cases each year in this country, 5000 to 10,000 individuals died (Edwards, 1990). After vaccination began, both morbidity and mortality decreased dramatically, although localized epidemics still occur (MMWR 1990; Olson, 1975). Only 5 to 10 patients died out of the 1200 to 4000 reported cases that occurred each year during the period of 1977 to 1987 (Edwards, 1990). However, over 50% of all reported pertussis cases and 70% of the mortality occurs in infants (Christie, 1989). In addition, in underdeveloped countries pertussis is still a major childhood problem due

to inadequacy of vaccination programs (Olson, 1975). The World Health Organization estimates that worldwide 600,000 unimmunized infants die annually (Edwards, 1990). Outbreaks of pertussis among adults are increasing even in the United States and may be due to a variety of causes including short-lived vaccine immunity, inadequate population immunity, or the identification of mild atypical pertussis as pertussis (Manclark, 1984; Christie, 1989). An analysis of maternal serum for antibodies against specific virulence factors of *B. pertussis* showed them to be low or absent, suggesting that adults are very susceptible to this disease (Edwards, 1990).

The use of the standardized whole cell *B. pertussis* vaccine combined with diphtheria and tetanus toxoids (DTP) in mass immunization programs has decreased with the increase in concerns over perceived vaccine adverse reactions. These adverse reactions run the gamut from redness, swelling, and/or pain at the injection site to non-febrile convulsions, infantile spasms, acute encephalopathy, and death. Public concern caused Japan to completely discontinue use of the DTP vaccine in 1981--with a concomitant increase in pertussis cases. An analysis of case reports in the literature, including the National Childhood Encephalopathy Study conducted in Great Britain, by Golden (1990) did not support the contention that this vaccine causes acute encephalopathy, infantile spasms, or sudden infant death syndrome. Golden stated that the increased risk of convulsions which can be attributed to the DTP vaccine does not produce brain injury or lead to epilepsy. In a study of children who had had pertussis as infants did not demonstrate any small airway defects, impaired lung function, alterations in antibody class

concentration, or increased incidence of respiratory infections compared to children without pertussis (Krantz, 1990).

However, public concern and potential litigation concerning the whole cell *B. pertussis* vaccine have encouraged the development of acellular vaccines. The two acellular vaccines currently available are the T type and the B type. The T type vaccine contains mostly filamentous hemagglutinin (FHA), toxoided pertussis toxin (PT) and some agglutinogens (see Physiology, Virulence Factors, and Genetics of *B. pertussis*). The B type vaccine contains equal amounts of FHA and toxoided PT and minute amounts of agglutinogens. Efforts to make a perfect vaccine and to assess the efficacy of these acellular vaccines in comparison with the DTP vaccine has lead to increased research on the genetics and virulence factors of *B. pertussis* and on the immune response of the host against these organisms.

Physiology, Virulence Factors, and Genetics of *B. pertussis*.

B. pertussis was first isolated from respiratory secretions of a clinical pertussis case in 1906 by Bordet and Gengou (Olson, 1975; Manclark, 1984). These bacteria were grown on an artificial medium consisting of potato starch infusion, glycerol, and defibrinated blood which has since been known as Bordet-Gengou (B-G) medium (Manclark, 1984). *B. pertussis* requires the presence of starch, charcoal (used in Regan-Lowe, i.e., charcoal, agar), or defibrinated blood to adsorb toxic unsaturated fatty acids from the medium. In addition, niacin or nicotinamide is also required for growth. Amino acids, rather than carbohydrates, are utilized as carbon and energy sources (Olson, 1975; Manclark, 1984). After 48-72 h under aerobic conditions, *B. pertussis*

produces punctiform, highly convex, glistening, translucent colonies with an entire margin on B-G medium (Olson, 1975; Manclark, 1984). Virulent strains produce a hazy zone of hemolysis while avirulent strains are nonhemolytic. Gram staining reveals pale staining Gram negative coccobacilli measuring 0.2-0.8 μm, roughly one-half the size of *E. coli.* Older cultures and cultures grown under adverse conditions demonstrate pleomorphism, filaments, and short chains (Manclark, 1984). These bacteria are nonmotile, nonsporulating, extremely fastidious obligate aerobes.

B. pertussis produces a battery of toxins and exoproducts which have been implicated as or shown to be important in establishing and in maintaining infection. Three virulence factors produced by *B. pertussis* directly cause inhibition or suppression of the host immune response: pertussis toxin, adenylate cyclase, and lipopolysaccharide. Pertussis toxin (PT; also known as pertussigen, lymphocytosis-promoting factor, histamine-sensitizing factor, and islet-activating protein) is an ADP-ribosyl transferase which irreversibly ribosylates the inhibitory guanine nucleotide-binding protein of eukaryotic adenylate cyclase (Katada, 1982). The resulting increase in intracellular cyclic AMP (cAMP) attenuates or eliminates signal transduction from cell surface receptors to internal mediator systems like insulin secretion (Ui, 1984). In addition, increased cAMP has been shown to inhibit various phagocytic functions (Weiss, 1986). By an apparently different mechanism, PT also alters cellular responsiveness to regulatory molecules via blockade of phosphatidyl inositol hydrolysis, arachidonate release, and calcium mobilization in various cells including polymorphonuclear leukocytes (PMNL), monocytes, macrophages, basophiles, bone marrow stem cells, and natural

killer cells (Spangrude, 1985; Weiss, 1986; Becker, 1986; Munoz, 1988). This alteration induces lymphocytosis and leukocytosis and inhibits chemotaxis of PMNL, lymphocytes and macrophages (Spangrude, 1985; Weiss, 1986; Becker, 1986; Munoz, 1988).

In addition to its toxic activities, PT acts as a carbohydrate-binding adhesin during the colonization phase of pertussis. Tuomanen and Weiss (1985) showed that *B. pertussis* transposon mutants deficient in PT did not adhere to human ciliated respiratory epithelial cells, suggesting that PT is an adhesion molecule. Relman *et al.* (1989) confirmed that PT is an adhesin of *B. pertussis* by showing that a mutant with a deletion of the PT operon *ptx* had reduced bacterial binding to ciliated respiratory epithelial cells. PT has been demonstrated to bind several distinct carbohydrates: galactose (Tuomanen, 1988); lactosamine (Tuomanen, 1988); and N-acetylneuraminic acid (Brennan, 1988). Ciliated respiratory epithelial cells have galactose- and lactosamine-containing molecules on their surfaces to which PT is believed to adhere (Tuomanen, 1988).

B. pertussis adenylate cyclase (BPAC) is a heat-stable calmodulin-activated protein similar to *Bacillus anthracis* edema factor (Leppla, 1984). This extracytoplasmic enzyme has been postulated to enter eukaryotic cells, to become activated by calmodulin, and to directly catalyze cAMP production (Confer, 1982). Although PT and BPAC cause the same net increase in cAMP, albeit by different mechanisms, the biological effects due to each toxin can be differentiated on the basis of response time. PT requires over 12 h to cause the same cAMP increase BPAC can cause in less than 1 h (Confer, 1982). BPAC induces diminished phagocyte chemotaxis, chemiluminescence

(an indirect measure of respiratory burst), and superoxide generation (a direct measure of respiratory burst), but does not alter phagocytosis by PMNL or monocytes (Confer, 1982; Weiss, 1986; Friedman, 1987; Galgiani, 1988). Alveolar macrophages treated with BPAC also demonstrate suppression of superoxide production as determined by reduction of ferricytochrome C (Confer, 1982).

The mechanism by which BPAC enters eukaryotic cells has not yet been ascertained. Since BPAC alters cAMP levels like many other toxins, it was thought to enter host cells via a binding subunit. No such subunit has been found either by trying to separate BPAC into subunits or by genetic analysis. Nor does BPAC gain entry into host cells via endocytosis (Gentile, 1988). There is some evidence that BPAC enters eukaryotic cells by means of direct contact between *B. pertussis* and the target cells (Hewlett, 1987). Mouallem *et al.* (1990) demonstrated that both purified BPAC and intact virulent *B. pertussis* increased cAMP in Chinese hamster ovary (CHO) cells. Cytochalasins B and D prevented CHO cell intoxication by intact bacteria but not by purified BPAC. Serum of two pertussis patients inhibited CHO cell intoxication, but not host cell invasion, by intact bacteria. The same sera had no effect on CHO cell intoxication induced by purified BPAC.

BPAC does appear to be a bifunctional protein in that it has toxic as well as weak hemolytic activity (Glaser, 1988). This hemolytic activity has been likened to *E. coli* hemolysin (Glaser, 1988), although it has not been demonstrated to be a true hemolysin. It is conceivable that the hemolytic activity of BPAC allows the entry of the molecule into the host cell where the toxic activity occurs. Bellalou *et al.* (1990) demonstrated that both the

hemolytic and the toxic activities must be present for full toxicity of the molecule (often called the cyaA protein). Deletion mutations of sections of the *cyaA* gene showed that hemolytic activity could be reduced and toxic activity eliminated without altering either the size of the protein or transport across the bacterial cell wall. In addition, Weiss *et al.* (1983) used a series of transposon-generated mutants with and without hemolysin production to study lethality in an infant mouse model. Their results strongly indicated that those mutants which retained hemolysin production were more lethal than those which did not. Finally, toxic and hemolytic activities could be restored in a $BPAC^{-}Hly^{-}$ mutant by complementation with plasmids bearing the intact *cya* operon (Glaser, 1988).

Recent studies by Rogel *et al.* (1991) and by Ehrmann *et al.* (1991) suggest that the hemolytic activity of BPAC occurred after a lag time of 40-80 min, although increased cAMP levels occurred almost immediately. In addition, the concentration of BPAC required for hemolysis was much higher than that necessary to increase cAMP levels. Hemolysis was independent of calcium availability while cAMP generation was dependent on extracellular calcium (Rogel, 1991). Hemolysis was enhanced, but cAMP generation was blocked, by exogenous calmodulin (Rogel, 1991; Ehrmann, 1991). These data strongly suggest that increased cAMP levels and hemolysis are separate, but related, events. Rogel *et al.* suggest that the enzymatic and hemolytic functions are mediated by different domains of BPAC. Osmotic protection of sheep erythrocytes by small molecular weight sugars suggest that BPAC forms a pore 3-5-fold smaller than that formed by *E. coli* hemolysin (Ehrmann,

1991). The relevance of this hemolytic activity in human pertussis has not been established.

Lipopolysaccharide (LPS) of *B. pertussis* is a unique endotoxin both structurally and functionally. It is composed of two immunochemically differentiable LPS's: LPS-I and LPS-II (LeDur, 1980; Ayme, 1980; Chaby, 1988). LPS-I contains lipid X, which possesses prototypical endotoxin activities, e.g., pyrogenicity, toxicity, induction of Schwartzman phenomena, and nonspecific immunity (Ayme, 1980; Manclark, 1984; Chaby, 1988). Lipid A possesses primarily potent antiviral and adjuvant activities (Ayme, 1980). The oligosaccharide chains have a variety of effects on different cell types including induction of interleukin-1 secretion by monocytes (Haeffner-Cavaillon, 1984; Chaby, 1988).

Filamentous hemagglutinin (FHA) is a non-fimbrial, cholesterol-sensitive protein with no demonstrated toxin activity (Manclark, 1984; Weiss, 1986). It is considered to be important in adherence to ciliated cells for several reasons. Strains of *B. pertussis* lacking FHA do not adhere well to human ciliated respiratory epithelial cells (Tuomanen, 1985). Anti-FHA antibodies have been shown to block adherence of *B. pertussis* to human ciliated epithelial cells and to protect mice (Oda, 1984; Tuomanen, 1985) and rabbits (Ashworth, 1982) from infection with *B. pertussis*. In addition, FHA induces production of increased titers of secretory IgA which could be correlated with clearance of *B. pertussis* from rabbit respiratory tracts (Ashworth, 1982). In mouse lung and tracheal colonization studies by Kimura *et al.* (1990), FHA was shown to be critical for initial colonization of the trachea, but not important for colonization of the lungs. Colonization of both

sites was greatly reduced by passive and by active immunization prior to aerosol challenge.

FHA has been shown to mediate adherence via two mechanisms: lectin-carbohydrate binding and protein-protein binding. Like PT, FHA binds galactose and lactosamine and molecules containing these carbohydrates on the surfaces of ciliated epithelial cells (Tuomanen, 1988). FHA also binds to CR3 (CD11b/CD18), the C3bi receptor on macrophages (Relman, 1990). The interaction of CR3 and C3bi involves an arginine-glycine-aspartic acid (RGD) sequence within C3bi. RGD sequences are found within extracellular matrix molecules like fibronectin and type I collagen. These sequences are recognized by integrin receptors on the surfaces of eukaryotic cells, which in turn cause alterations in cell function. Adhesive leukocyte surface proteins are also integrins, including CD3, LFA-1, and p150,95. p150,95 regulates the diapedesis of PMNL through endothelial cells into tissues (Ruoslahti, 1987). RGD sequences occur at two sites within FHA (Relman, 1989).

Fimbriae or pili are long filamentous structures protruding from the bacterial surface which are also believed to be important in adherence. Originally fimbriae were called agglutinogens and a system of six serotypes was established based on agglutination of type-specific antisera (Eldering, 1969). They are structurally, chemically, and antigenically distinct from FHA (Manclark, 1984; Cowell, 1986; Zhang, 1985; Friedman, 1988). The importance of fimbriae in pathogenesis is indicated by the protection of mice immunized with fimbriae from aerosol challenge by *B. pertussis* (Zhang, 1985). In addition, the demonstration of high anti-fimbriae titers has been linked to protection of individuals from pertussis (Manclark, 1984).

Dermonecrotic toxin (also known as heat-labile toxin, mouse-lethal toxin, or lienotoxin) is one of only two toxins produced by all four *Bordetella spp.* (Gentry-Weeks, 1988; Endoh, 1990). It is a polypeptide complex which has been suggested to be located partially on the bacterial cell wall, but mainly in the cytoplasm (Livey, 1984; Manclark, 1984). Although its mechanism of action has yet to be established, dermonecrotic toxin has been shown to cause vasoconstriction leading to local ischemia, leukocyte migration, and petechial hemorrhage possibly by the inhibition of Na^{+}-K^{+} ATPase (Nakase, 1985; Weiss, 1986; Friedman, 1988). Dermonecrotic toxin also causes splenic atrophy when administered intravenously, skin lesions when administered intradermally, contraction of cultured smooth muscle cells, and inhibition of osteogenesis of a cultured osteoprogenitor-cell line (Endoh, 1990; Horiguchi, 1991). The kind of skin lesions caused by dermonecrotic toxin depends on the animal injected. Ischemic lesions develop in rabbits and guinea pigs, while suckling mice develop hemorrhagic lesions and adult mice are refractory to the toxin (Endoh, 1990). Endoh *et al.* (1990) have shown that the skin of adult mice contains long-chain unsaturated fatty acids which inhibit the action of dermonecrotic toxin by an as yet undetermined mechanism.

Tracheal cytotoxin is the other toxin produced by all *Bordetella spp.* It toxic only to ciliated cells. It has been shown to inhibit DNA synthesis without altering RNA and protein synthesis in hamster tracheal epithelial cell cultures and to cause cellular damage and ciliostasis to hamster tracheal epithelial cells (Goldman, 1986; Rosenthal, 1987). Maximum inhibition of ciliary activity required 1 μM tracheal cytotoxin, the concentration produced in *B. pertussis* culture supernatants (Cookson, 1989). These biological activities cannot be

replicated using purified LPS from either *B. pertussis* or *E. coli*, emphasizing that this toxicity is due exclusively to tracheal cytotoxin (Cookson, 1989). Wilson *et al.* (1991) used nasal epithelial biopsy specimens from children with pertussis, normal children, and normal adults to study the effects of tracheal cytotoxin. Neither culture filtrates nor purified tracheal cytotoxin affected ciliary function. However, both caused extrusion of ciliated cells, an increase in sparsely ciliated cells, and toxic changes in some cells leading to cessation of ciliary beating within 90 h in a dose-dependent manner.

Biochemically, tracheal cytotoxin appears to be a single low molecular weight glycopeptide composed of glucosamine, muramic acid, alanine, glutamic acid, and diaminopimelic acid (Cookson, 1989). This composition is typical of *B. pertussis* peptidoglycan and the toxin is believed to be released as fragments during turnover of the cell wall matrix (Goldman, 1986; Rosenthal, 1987; Cookson, 1989). The muramic acid composition of tracheal cytotoxin suggests it is a member of the muramyl peptide family, which has a variety of biological effects including pyrogenicity, adjuvanticity, arthritogenicity and stimulation of leukocytes to produce interleukin-1 (Cookson, 1989). Another member of the muramyl peptide family is a dissacharide peptide released by *Neisseria gonorrhoeae* which also causes ciliated-cell-specific damage (Melly, 1984).

The newest *B.-pertussis* virulence factor defined thus far is pertactin, a nonfimbrial 69-kDa protein present on the surfaces of all virulent *B. pertussis* strains (Shahin, 1990; Brennan, 1988). An antigenically similar protein is found in *B. bronchiseptica* and *B. parapertussis* (Shahin, 1990). Pertactin is involved in adherence of *B. pertussis* to eukaryotic cells via an RGD

sequence (Leininger, 1991). A mutant deficient in pertactin bound to CHO cells and HeLa cells much less efficiently than the parent strain (Leininger, 1991).

B. pertussis undergoes both phase variation and phenotypic modulation. Virulent bacteria spontaneously lose their virulence at a frequency of 1 per 10^3 to 10^6 organisms; reversion to full virulence occurs at very low frequency (Weiss, 1984a). Avirulent phase variants produce only LPS and tracheal cytotoxin (Weiss, 1986). The *bvgAS* operon (for *Bordetella* virulence locus, also known as *vir*) encodes a trans-acting positive inducer that turns on the genes encoding PT, BPAC, dermonecrotic toxin, FHA, and several other outer membrane proteins (Weiss, 1984a; Stibitz, 1988). This inducer appears to regulate these virulence factor genes at the level of transcription (Roy, 1989). It is unknown if repressing this operon allows other genes to be expressed (Weiss, 1986). The expression of virulence factors is negatively regulated by low temperature (< 37°C, i.e., body temperature) or presence of high amounts of $MgSO_4$ or nicotinic acid (Melton, 1989; Roy, 1989). Proteins expressed by this operon are believed to be two-component signal transduction proteins which respond to environmental stimuli; similar proteins have been found in many other bacteria (Arico, 1989; Miller, 1989). The 23-kilodalton BvgA appears to be homologous to response regulator molecules while the 102-kilodalton BvgS appears to be homologous to sensor-transmitter molecules (Miller, 1989). Transcription regulation of the *bvgAS* operon appears to be self-activating like *glnALG*, the nitrogen regulation operon of *E. coli* (Roy, 1990). Self-activation is also inhibited by low temperature, high $MgSO_4$ levels, or high nicotinic acid levels.

Clinical Pertussis.

The incubation period of pertussis is generally 7 days, with a range of 6 to 20 days after exposure. Initial symptoms resemble those of a typical cold: rhinorrhea, conjunctival edema and tearing, sneezing, and occasional mild coughing. A low grade fever (approximately 101°F) may be present. This catarrhal stage lasts 1-2 weeks at which time *B. pertussis* can be isolated from up to 90% of nasopharyngeal specimens (Olson, 1975; Manclark, 1984; Weiss, 1986). In neonates, the catarrhal phase may be shortened or absent (Christie, 1989). Nonproductive catarrhal phase coughing gradually increases in severity and frequency to become productive paroxysmal stage coughing. Paradoxically, after the initiation of the paroxysmal stage, the isolation rate of *B. pertussis* drops even without the use of appropriate antibiotics (Weiss, 1986).

The 1-4 week paroxysmal stage is characterized by severe coughing seizures that begin suddenly without anticipation and often end in vomiting of stomach contents and abundant viscous mucus. Inspiration at the end of a paroxysm generally produces the characteristic whoop, although not every patient whoops nor does whooping occur with every paroxysm (Olson, 1975; Manclark, 1984). Neonates and very young children are more likely to display choking, apnea, and cyanosis than whooping while immunized children and adults may develop only a common cold or a mild bronchitis (Robertson, 1987; Friedman, 1988; Christie, 1989). Despite the severe respiratory inflammation, wheezing while breathing does not occur even in asthmatic patients (Olson, 1975). Physiological effects characteristic of the paroxysmal stage include exhaustion, dehydration, malnutrition, conjunctival and petechial

hemorrhages, herniae, emphysema, and pneumothorax (Olson, 1975; Manclark, 1984). Bradycardia and unresponsiveness are also typical of neonatal pertussis (Christie, 1989). Lymphocytosis, leukocytosis, and hypoglycemia are also typical (Weiss, 1986), although in neonates lymphocytosis is rare (Christie, 1989). Fever is absent in the absence of complications, most of which develop during the paroxysmal stage.

The most serious complications of pertussis are encephalopathy, secondary bronchopneumonia, collapsed lung(s), and prolonged hypoxia which may lead to irreversible brain damage or death (Manclark, 1984; Weiss, 1986). Convalescence from uncomplicated pertussis is slow, gradual, and of long duration. Spontaneous paroxysms may take six months to disappear; isolated coughing bouts may recur with any respiratory infections for up to two years after acute pertussis (Olson, 1975; Manclark, 1984).

The importance of the virulence factors is apparent upon analysis of the pathology of pertussis as it is currently perceived (Olson, 1975; Manclark, 1984; Weiss, 1986). Once the respiratory tract is inoculated by droplet infection, *B. pertussis* adheres to ciliated epithelial cells by means of FHA, surface-bound PT, fimbriae, and possibly pertactin, where multiplication begins. Production of toxins and other virulence factors is initiated. Within 24 h of infection, cilia die and are shed; within a few days the naked epithelial cells themselves are extruded. This loss may be due to the multiplication of the bacteria, the effects of tracheal cytotoxin or both (Olson, 1975; Manclark, 1984; Weiss, 1986).

Peribronchial lymphoid hyperplasia develops followed by necrotic inflammation of the tracheobronchial mucosa, possibly the result of the actions

of tracheal cytotoxin or dermonecrotic toxin or both (Olson, 1975; Manclark, 1984). It seems likely that PT and BPAC are important in this facet of disease since cholera toxin, which causes a similar increase in cAMP, induces massive fluid and mucus secretion into the gastrointestinal tract (Weiss, 1986). However, there is no current evidence to support the effects of either toxin on ciliostasis, epithelial cell death, or other related pathological changes (Manclark, 1984). Still, chemotaxis of phagocytic cells into the respiratory tract as well as their function is inhibited by PT and BPAC; the function of resident alveolar macrophages is similarly inhibited (Olson, 1975; Manclark, 1984; Weiss, 1986). Significantly increased mucus secretion and extensive inflammation of bronchial tissues leads to respiratory stasis from nasopharynx to alveoli. Eventually, bronchiolar obstruction and anoxia occur (Olson, 1975). The potential for secondary infection or other complications is high due to tissue damage and a malfunctioning immune response. In addition to pathological effects in the respiratory tract, pertussis has systemic effects with characteristic symptoms. Lymphocytosis, leukocytosis, aberrant glucose homeostasis, attenuation of febrile and inflammatory response, and possibly neurological effects are caused by PT and BPAC (Olson, 1975; Manclark, 1984; Weiss, 1986).

The mechanism of recovery and immunity from pertussis has not been completely ascertained, although it is generally believed to be at least partially dependent upon antibody-mediated clearance since production of certain specific antibodies appears to provide protection from infection and re-infection (Olson, 1975; Manclark, 1984; Friedman, 1988). A variety of assays have been used to determine serological response to infection as well as

vaccination. Still, there is no clear, consistent image of the mechanisms of bacterial clearance or of protection from infection (Olson, 1975; Manclark, 1984; Friedman, 1988). This is easily understandable considering the number of virulence factors *B. pertussis* has and the knowledge that specific antibodies do not appear until the third or fourth week of disease (Olson, 1975; Manclark, 1984). Serum bactericidal activity, dependent on either human or animal complement and antibody, has been shown to be very effective in killing *B. pertussis* (Dolby, 1965; Dolby, 1965; Brighton, 1969; Aftandelians, 1973). Assuming that all invading bacteria are killed by serum components, what role, if any, do phagocytic cells fulfill in bacterial clearance beyond that of scavenger?

Interactions of *B. pertussis* With the Humoral Immune Response.

Most of the literature dealing with immune responses against *B. pertussis* was done during or before the 1970's. These studies used human and animal phagocytes and sera in combination with virulence factors in various stages of purification. Results of early research may or may not be accurate for a variety of reasons, including the lack of an ideal animal model of pertussis. Although animals can be experimentally infected with *B. pertussis* both intranasally and intracerebrally, the human symptomatology is not fully reproduced (Olson, 1975; Manclark, 1984; Weiss, 1986). Primates are the only known animals that develop the paroxysmal cough and abundant mucus production that are hallmarks of human pertussis (Weiss, 1986). Rats cough and accumulate fluid in the lungs, but do not produce abundant mucus (Weiss, 1986). Rabbits develop the respiratory, but not the systemic, symptoms

(Ashworth, 1982). Mice infected either intranasally or by aerosol develop many pertussis symptoms which are fatal to infant mice, but not to adult mice (Pittman, 1980). In addition, animals are typically colonized by *Bordetella bronchiseptica* leading to the generation of cross-reactive antibodies to *B. pertussis* making interpretation of results difficult.

Some of the earliest studies on humoral immune response were those of Dolby (1965), Dolby and Vincent (1965), and Brighton *et al.* (1969) on serum bactericidal activity against *B. pertussis*. These studies showed that human and mouse serum bactericidal activity was directly proportional to the amount of complement present, with bacterial strain and individual serum source being of lesser relevance. Maximum serum killing required no more than 30 min. Removal of complement by heating sera in a 56^{0}C water bath for 30 min completely eliminated bactericidal activity regardless of serum source or bacterial strain. Exogenous addition of complement returned cidal activities to their original levels. Complement alone had no effect on bacterial viability, suggesting that IgG or IgM must be required for serum bactericidal activity. An electron micrograph by Brighton *et al.* (1969) showed holes in the surface of *B. pertussis* characteristic of complement lysis. Later studies by Aftandelians and Connor (1973) confirmed the presence and demonstrated the development of serum bactericidal activity in convalescing pertussis patients. Complement-dependent bactericidal antibodies ("bactericidins") were present at some time during the illness of all patients and were detectable in many up to 40 weeks after acute disease. "Bactericidins" were primarily IgG rather than IgM in the one patient serotyped.

Antibody responses generated by specific virulence factors were assessed once the latter began to be isolated, purified, and characterized. Interest in anti-*B. pertussis* response has followed two directions. First, antibodies generated by immunization with specific virulence factors may be protective against lethal infection in animals and against pertussis in humans. This is the rationale for the development of acellular vaccines. Second, antibodies against specific virulence factors generated by infection or vaccination with *B. pertussis* may be identified by serological methods. This is the rationale for the development of commercial serological tests with clinical microbiology applications.

In studies of animal antibody responses against virulence factors, mice have been protected from intracerebral, intraperitoneal, and aerosol infection of *B. pertussis* by treatment with either PT-toxoid or anti-PT antisera (Cowell, 1982; Sato, 1981; Sato, 1984). Once the two subunits forming PT were isolated, monoclonal antibodies against the A subunit protected mice from intracerebral and aerosol challenge (Sato, 1984). Attempts to protect mice by the B subunit or by antibodies against it has given conflicting results (Sato, 1984; Arciniega, 1987). Immunization of mice with FHA prevented aerosol infection; administration of anti-FHA sera did not prevent infection (Oda, 1984; Sato, 1984). However, more recent expriments have shown that mice can be succesfully protected by passive immunization (Kimura, 1990). Increased anti-FHA secretory IgA has been directly correlated with clearance of *B. pertussis* from rabbit respiratory tracts (Ashworth, 1982). Immunization of mice with anti-BPAC monoclonal antibodies prevented aerosol infection by *B. pertussis* (Brezin, 1987). Immunization of neonatal mice with pertactin or

anti-pertactin monoclonal antibodies protected against aerosol infection (Shahin, 1990).

In studies of human antibody responses against virulence factors, infected children demonstrated high anti-PT titers, while vaccinated children did not (Winsnes, 1985). However, in another study of vaccinated children there was a direct correlation between anti-PT serum titer and immunity (Granstrom, 1985). In addition, immunoblotting studies confirmed the production of anti-PT A subunit antibodies due to infection or immunization (Thomas, 1989). Anti-FHA antibodies have been shown to block adherence of *B. pertussis* to human ciliated epithelial cells (Tuomanen, 1985). Antibodies against FHA and LPS are most persistent in humans after both infection and vaccination (Winsnes, 1985; Redd, 1988; Thomas, 1989). Immunization of children with DTP vaccine induces production of antibodies against FHA and PT (Thomas, 1989). In addition, immunization of people with both FHA and PT has been demonstrated to have a synergistic effect and so are present together in the acellular vaccines (Sato, 1984; Sato, 1984; Marchitto, 1987). Recently, high titers of anti-BPAC IgG were demonstrated in patients with pertussis (Farfel, 1990). These IgG antibodies did not block enzymatic activity of purified BPAC or prevent the ability of BPAC to enter human lymphocytes *in vitro*. Anti-pertactin antibodies have been found in pertussis patients or vaccinated individuals (Thomas, 1989; Shahin, 1990).

Interactions of *B. pertussis* With Phagocytic Cells.

Some of the earliest studies on the phagocytic cell immune response to pertussis were done using animal models. Gray and Cheers (1967)

demonstrated that "immunological complaisance", a phenomenon characteristic of mouse pulmonary tuberculosis, occurred in mouse pertussis infections. The authors (1967; Cheers, 1969) used histopathology of lung sections, lung impression smears, and colony plate counts of homogenized lung tissue to describe this phenomenon. By 7 days after intranasal administration, *B. pertussis* were visible free in the bronchiolar mucus and in association with alveolar macrophages. By 11 days, 1 to 5 bacteria were seen per alveolar macrophage. By 14 days, the PMNL invasion of the bronchioles was accompanied by an abrupt tenfold drop in viable bacteria and an abrupt disappearance of free bacteria visible in the mucus. After 14 days, the number of PMNL rapidly decreased, but alveolar macrophages contained a steady state level of viable *B. pertussis* maintained at < 1% of the pre-immune peak. This steady state phase the authors called "immunological complaisance". Bactericidal activity recurred during the complaisant phase if this steady state level of *B. pertussis* was exceeded. The complaisant phase was followed by another intense period of bactericidal activity leading to complete recovery by 7 weeks post-inoculation.

Hypothesizing cellular immunity was adversely affected, functional analysis of peritoneal macrophages from complaisant versus normal mice against viable *B. pertussis* was conducted (Cheers, 1969). Peritoneal macrophages from complaisant mice were not more phagocytic than those from normal mice, either *in vivo* or *in vitro*, but they displayed increased killing activity. Serum dilution studies indicated an eightfold higher opsonic titer in complaisant mouse sera than that of normal sera. These opsonins were heat stable, unlike those of normal mouse serum suggesting the former were not

complement proteins nor were they dependent on complement. However, opsonization of *B. pertussis* with normal or complaisant sera did not alter phagocytosis by peritoneal macrophages suggesting no benefit of the increased opsonin levels. Gray and Cheers (1969) showed that the increased killing activity of complaisant peritoneal macrophages disappeared as organismal clearance and convalescence occurred. Clearance and convalescence coincided with the production of protective levels of antibody by the sixth week of infection. The protective antibody, which was not further characterized, increased phagocytosis of *B. pertussis* by normal and convalescent macrophages, but had no effect on killing. No additional explanation was provided.

More recent studies on phagocytic cell responses have been conducted using specific virulence factors of *B. pertussis*. Studies performed by Confer and Eaton (1982), Friedman *et al.* (1987), and Galgiani *et al.* (1988) have demonstrated that BPAC inhibited human PMNL chemotaxis and chemiluminescence (CL) induced by particulate stimuli, without altering phagocytosis. However, the particulate stimuli used were zymosan, *S. aureus*, or *Candida glabrata* rather than intact *B. pertussis*. The fact that phagocytosis of each stimulus was unchanged by BPAC treatment of PMNL suggests that CL inhibition is a separate phenomenon from phagocytosis. Confer and Eaton (1982) reported the ability of BPAC to inhibit PMNL bactericidal activity against *S. aureus*. Studies by Spangrude *et al.* (1985) and Becker *et al.* (1986) have shown that PT also inhibits chemotaxis, lysosomal enzyme production, and superoxide production in human and rodent PMNL and lymphocytes.

Unfortunately, interactions between phagocytic cells and individual virulence factors described by these authors provide at best an incomplete assessment of phagocyte response to the intact bacteria. The killed whole cell pertussis vaccine itself has been demonstrated to alter many immune responses, including induction of leukocytosis and lymphocytosis and inhibition of chemotaxis in rodents (Munoz, 1988). These alterations are believed to be caused by PT or LPS, although the literature contains no information as to the possibility that BPAC might participate in such modifications (Munoz, 1988).

In order to answer some of the questions regarding the importance of specific virulence factors of *B. pertussis* in its pathogenesis, Weiss *et al.* (1983) used transposon Tn5 mutagenesis to create a series of mutants, each deficient in either PT, FHA, BPAC, hemolysin or in all four. They also isolated two spontaneous mutants: an FHA$^-$ mutant and an avirulent mutant (Table 2-1 in Methods and Materials). Weiss *et al.* (1984b) determined the effects of the transposon mutations on virulence in the infant-mouse model. Bacteria were administered intranasally and dilution plate counts of minced lung tissue were made. The avirulent BP326 and BP347 mutants did not cause a lethal infection even at very high doses. Surprisingly, BP353 (FHA$^-$) was as lethal as parent strain BP338, suggesting that FHA is less important in establishing pertussis in the mouse than had previously been considered. On the other hand, BP357 (PT$^-$) was much less lethal than BP338, emphasizing the importance of PT in pathogenesis. The importance of BPAC and hemolysin remained clouded because although BP349 (BPAC$\pm$Hly$^-$) was more lethal than BP357, BP348 (BPAC$^-$Hly$^-$) did not cause a lethal infection. However,

BPAC activity was reduced in both mutants. Weiss *et al.* (1984b) also observed that sublethal infections induced persistence of the mutants in mouse lung tissue, although their sample size was quite small.

Adherence of these mutants to human ciliated epithelial cells was demonstrated by Tuomanen and Weiss (1985). Tracheal epithelial cells, collected during bronchoscopy, were cultured and infected with the Tn5 mutants. Avirulent strains BP326 and BP347 were nonadherent and wild type BP338 was very adherent. Interestingly, BP357 (PT^-) was more adherent than BP338, suggesting that the strain may be unable to transport PT across the membrane leaving it membrane-bound. This suggestion has been supported by Marchitto *et al.* (1987) in that the A subunit is synthesized by BP357, but is not secreted due to the Tn5 inactivation of the B subunit structural genes. These data support the contention that PT also functions as an adhesin. Of the other strains tested by Tuomanen and Weiss (1985), only BP349 ($BPAC^{\pm}Hly^-$) was moderately adherent. The authors concluded that the loss of adherence was associated with the lack of secretion of FHA or PT.

To further assess the association of specific virulence factors with ability to cause disease, Weiss and Goodwin (1989) made another group of virulence factor mutants using Tn5*lac*. Tn5*lac* is the Tn5 transposon containing a promoterless ß-galactosidase gene; ß-galactosidase is expressed only when Tn5*lac* is inserted into a functional gene. Bacteria were administered intranasally to infant mice and dilution plate counts of minced lung tissue were made. In order to test how fast virulence factors could be turned on, BP338 was grown in $MgSO_4$ prior to inoculation. There was no significant change in the lethal dose, suggesting that virulence genes can be

turned on before the host can eliminate the bacteria. As seen with the Tn5 mutants, a PT$^-$ mutant was much less lethal than the wild type. Two BPAC$^-$Hly$^-$ mutants were unable to establish a lethal infection even at high dose. Mutants deficient in dermonecrotic toxin or FHA were able to cause lethal infection equal to that caused by the wild type. A mixture of BP348 (BPAC$^-$Hly$^-$) and BP357 (PT$^-$) was administered to determine if phenotypic complementation was possible. The mixture was unable to initiate a lethal infection below 10^6 CFU, the lethal dose for BP348 alone. However, both strains could be isolated individually for up to 35 days post-inoculation. Interestingly, BP357 was recoverable for a longer period of time if administered with BP348 than alone, suggesting that some type of interaction dependent on BP348 was occurring.

Additional studies were performed using infant mice infected intranasally with sublethal levels of *B. pertussis* mutants (Goodwin, 1990). Administration of 500 BP338 resulted in rapid multiplication to a peak of 10^7 bacteria and persisted until day 40. Forty-eight h after administration of 10^7 BP347 no bacteria were recovered from the lungs. Again, an FHA$^-$ mutant was as virulent as BP338. Low dose inoculation of BP357, which does not readily cause lethal infection of infant mice, showed multiplication similar to BP338 until day 10. After day 10, no further multiplication occurred and the bacteria were cleared in 25-30 days. Inoculation of a higher dose of BP357 resulted in the persistence of greater numbers of bacteria which required 40 days to clear. This suggests that PT is not important in the early stages of pertussis, but is critical in the later stages. Low dose inoculation of BP348, which also does not readily cause lethal infection, was cleared by day 10.

However, inoculation of a higher dose of BP348 resulted in multiplication and the infection persisted until day 50. This suggests that BPAC is critical in the early stages of pertussis and that there is a threshold of bacterial numbers necessary to prevent clearance by the immune response (Goodwin, 1990).

In these reports using mutants to infect infant mice, the authors conclude that the bacteria must be invading lung cells in order to persist up to 40 or 50 days (Weiss, 1989; Goodwin, 1990). However plausible their conclusion might sound, no proof was presented. In an early report that would link presence of bacteria with invasion, Hopewell *et al.* (1972) used transmission EM to examine the effects of intracerebral inoculation of *B. pertussis* into mice. The classical method for assessing potency of pertussis vaccines is to inoculate *B. pertussis* intracerebrally into mice. Hopewell *et al.* observed bacteria between the microvilli and within cytoplasm of the ependymal cells lining the cerebral ventricles. Ependymal cells are ciliated cells which have some phagocytic activity. These findings suggest that either the bacteria were phagocytosed or were actively invasive.

Ewanowich *et al.* (1989a; 1989b) examined the ability of *B. parapertussis* and *B. pertussis* to invade HeLa 229 cells. *B. parapertussis* is similar to *B. pertussis* except that it does not produce PT and causes a lower incidence of human infection. Invasion of HeLa cells by *B. parapertussis* does not appear to occur via receptor-mediated endocytosis, although entry is microfilament-dependent. Anti-FHA IgG was unable to prevent invasion of these organisms, indicating that other adhesins are important in this system. The numbers of viable *B. parapertussis* recovered after 5 h of coincubation with HeLa cells were comparable to the numbers of invasive *Salmonella*

hadar and *Shigella flexneri*. However, while *B. parapertussis* invades and survives within HeLa cells, they do not multiply (1989a). In general, the invasive behavior of *B. pertussis* is similar to that of *B. parapertussis* (1989b). However, invasion of HeLa cells could be inhibited by anti-FHA IgG, anti-whole cell *B. pertussis* and anti-LPS-A.

Invasion by the Tn5 mutants were also assessed in this system. BP353 and BP357 were much less invasive than the wild type strain BP338. In contrast, BP348 and BP349 were more invasive than BP338. The fact that BP347 was unable to invade HeLa cells indicates that one or more virulence factors is crucial for invasion or subsequent survival in this system.

Additional studies of HeLa 229 cells invasion were conducted by Lee *et al.* (1990) to assess the impact of various genetic alterations on invasion. Invasion was shown to be time and temperature dependent. HeLa cell invasion by two wild type strains could be decreased or eliminated by growth in the presence of the environmental inhibitors nicotinic acid, $MgSO_4$, and growth at 27°C. Interestingly, loss of BPAC (BP348), PT (BP357), or FHA (BP353) did not prevent invasion. There was no explanation for the apparent difference observed in this study and that by Ewanowich *et al.* (1989b). The avirulent mutant (BP347) did not invade HeLa cells until a plasmid containing the *bvg* region was inserted; 49% of the bacteria complemented by this plasmid were able to invade. On the other hand, two *bvg*⁻ mutants were able to invade HeLa cells. One of these mutants was created by UV-irradiation of a wild type strain and the other was created by insertion of a plasmid in the *bvg* locus. Both were nonhemolytic and PT^- and produced small amounts of full-length FHA and pertactin. The authors hypothesize that excess *bvgA* is

produced, directly turning on production of FHA and pertactin; this type of activation has been shown to occur in *E. coli* (Roy, 1989).

It is clear that fundamental questions regarding host defense and bacerial clearance in pertussis remain unanswered. Studies on infant mice and on HeLa cells strongly suggest that *B. pertussis* can invade and survive inside eukaryotic cells. Intracellular survival of *B. pertussis* might explain many unanswered questions regarding pertussis. However, it is important that the scenario present in infant mouse and in tissue culture models be related to the scenario in human pertussis. If light can be shed on the mechanism by which the human host and *B. pertussis* interact, an intracellular habitat may explain the extreme longevity of pertussis as well as the lack of an identifiable carrier or vector. Therefore, the primary goal of this research was to identify basic concepts of host defense against *B. pertussis* using solely human PMNL, sera, and antibody. In addition, if the relevance of the various *B. pertussis* virulence factors from the perspective of host defense can be determined, the development of a more effective and safer vaccine might be enhanced. Using a series of *in vitro* assays the following questions were addressed. First, do PMNL kill *B. pertussis* as effectively as they kill *E. coli*? If not, how might *B. pertussis* avoid PMNL bactericidal activities? Second, using a series of transposon and deletion mutants, which specific virulence factors play a role as antigens in antibody-induced phagocytosis by PMNL? Third, do these virulence factors aid *B. pertussis* to resist PMNL bactericidal activity?

CHAPTER II

METHODS AND MATERIALS

Bacterial Strains and Media.

Staphylococcus aureus, a clinical isolate, was used as a control for early phagocytosis assays and for EM studies because of its very large size. *Escherichia coli*, strain Bi 161-42, was used as a control for all assays except EM. Stocks of both were maintained in a skim milk-glycerol mixture at -70°C. A stock suspension of 1.5×10^8 *S. aureus*/ml was killed by 60 min incubation in a 56°C water bath, aliquoted, and stored at -20°C until used. *E. coli* was grown overnight on an LB agar plate before being used to inoculate a small flask containing trypticase soy broth. The flask was incubated in a 37°C gyratory incubator and checked periodically until an $OD_{650} = 0.2$ was attained. For respiratory burst and phagocytosis assays, the suspension was diluted to a final concentration of 1.5×10^7 *E. coli*/ml. For PMNL killing assays, the suspension was diluted to a final concentration of 2.5×10^5 *E. coli*/ml.

The strains of *B. pertussis* used in this research were three wild type strains and a series of spontaneous, deletion-generated or transposon-generated mutants (see Table 2-1). Stocks of each strain were maintained in a 50% Stainer-Scholte medium-50% glycerol mixture at -70°C. Maintenance of *B. pertussis* strains in the virulent phase was verified by the presence of zones of hemolysis around isolated colonies grown on Bordet-Gengou agar (Weiss, 1986). Fresh cultures were prepared by inoculating charcoal agar (Difco Laboratories, Detroit, MI) with 50 µl of thawed stock. Plates were

incubated at 37°C in a moist environment for 2-3 days before use. The lawn from a plate was harvested with a sterile dacron swab, suspended in phosphate buffered saline (PBS) and centrifuged at 900 x g to wash, and resuspended in Stainer-Scholte medium at an $OD_{650} = 0.1$. For respiratory burst and phagocytosis assays, the suspension was used directly at a final concentration of 1.5×10^7 *B. pertussis*/ml. For killing assays, the suspension was diluted to a final concentration of 2.5×10^5 *B. pertussis*/ml.

Normal Human Sera (NS) and Human Anti-*B. pertussis* Antibody (HAPA).

Autologous NS were collected from clotted venous blood of PMNL donors, held on ice, and used for opsonization. Individuals who donated blood had been vaccinated as children; none had had pertussis. HAPA (Tosuman Berna) was purchased from Swiss Serum and Vaccine Institute, Bern, Switzerland. HAPA is a purified immunoglobulin fraction of pooled human sera obtained from donors demonstrating high agglutinin titers against *B. pertussis.* HAPA was prepared at an 18% concentration (w/v) in PBS and was stored at -20°C. A 1:100 dilution of HAPA, the concentration shown experimentally to induce optimum *B. pertussis* phagocytosis (data not shown), was used for opsonization. An IgG fraction was prepared from the NS of four individuals demonstrating high anti-whole cell *B. pertussis* titers by ELISA. The NS were pooled and delipidated using the procedure of Neoh *et al.* (1986) and the IgG fraction isolated following the procedure of McKinney and Parkinson (1987). Protein concentrations of the IgG fraction and HAPA were determined using the Pierce BCA protein assay (Rockford, IL) according to the

package insert. Guinea pig complement was from Gibco (Gibco Laboratories, Grand Island, NY).

Enzyme-linked immunosorbent assays (ELISAs) were performed on all NS, IgG fraction, and HAPA to determine anti-whole cell *B. pertussis* titers using a modification of the procedure by Madore *et al.* (1986). Briefly, microtiter plates (Immulon 2, Dynatech Laboratories, Chantilly, VA) were coated with each *B. pertussis* strain at $OD_{650} = 0.1$, suspended in 0.1 M carbonate buffer, pH 9.6, at 4°C overnight. The plates were blocked with 5% skim milk in 50 mM Tris/50 mM NaCl/0.05% Chaps (TN-Chaps; Research Organics, Inc., Cleveland, OH). Dilutions of NS, IgG fraction, and HAPA were made in TN-Chaps, added to wells in 100 µl aliquots, and incubated at 37°C for 3 h. The presence of anti-*B. pertussis* antibodies were detected by the addition of 100 µl horseradish peroxidase-conjugated goat anti-human IgG, goat anti-human IgA, and goat anti-human IgM antisera (all from Cappel, Malvern, PA) and overnight incubation at 4°C. The color reaction was developed with the addition of a 3,3',5,5'-tetramethylbenzidine (TMB; Calbiochem, San Diego, CA) reaction mixture, stopped with 1 N HCl, and read on a Titertek multiscan photometer at 450 nm.

The presence of complement in NS and HAPA was assessed by determination of the presence of C3 and C4 by rate nephelometry (Sternberg, 1977). These assays were performed by Dr. Paul Finley, Dept. of Pathology, University of Arizona Health Sciences Center.

Preparation of Rabbit Anti-avirulent Antiserum (RAAA).

A New Zealand white rabbit was immunized with heat-killed BP326 mixed with Freund's complete adjuvant (Difco) after collection of control serum. The vaccination was repeated 21 days later. On day 28, serum was collected and heat-killed BP326 without adjuvant was inoculated. Since we felt that the antibody titer was not increasing as expected, on days 35 and 49 the rabbit was boosted with heat-killed BP326 + 0.8% aluminum hydroxide (Alhydrogel; Accurate Chemical and Scientific Corp., Westbury, NY). ELISAs and phagocytosis assays were conducted with serum collected on the day 57. Although we tried to maintain the rabbit to have a continuous supply of antiserum, the rabbit developed incurable snuffles and was exsanguinated by cardiac puncture 3 months after vaccination began.

ELISAs on rabbit antisera were performed using a modification of a standard procedure (Winston, 1987). Briefly, microtiter plates were coated with BP338, BP347, and BP326 at $OD_{650} = 0.1$, suspended in 0.1 M carbonate buffer, pH 9.6, at 4°C overnight. The plates were blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-Tween). Dilutions of serum from different collection dates were made in PBS-Tween, added to wells in 100 μl aliquots, and incubated at 37°C for 3 h. The presence of anti-*B. pertussis* antibodies were detected by the addition of 100 μl horseradish peroxidase-conjugated Protein A (Zymed, So. san Francisco, CA) and overnight incubation at 4°C. The color reaction was developed with the addition of a 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS; Aldrich Chemical Co., Milwaukee, WI), reaction mixture, stopped with 5% SDS, and read on a Titertek multiscan photometer at 405 nm. Anti-whole

cell BP326 titer of RAAA was 1:400 by this method. No attempt was made to determine the immunoglobulin class(es) present by ELISA.

Human Polymorphonuclear Leukocyte (PMNL) Isolation.

PMNL were isolated daily from venous blood treated with heparin according to the protocol of Friedman *et al.* (1987). Briefly, PMNL were separated from serum, monocytes, and lymphocytes by Ficoll-Hypaque gradient centrifugation. PMNL were harvested and washed with saline; contaminating erythrocytes were hypotonically lysed. After washing in Hanks balanced salts solution containing 0.1% gelatin (gel-HBSS), PMNL were resuspended in gel-HBSS at a concentration of 2 x 10^6 PMNL/ml for respiratory burst and phagocytosis assays at 1 x 10^7 PMNL/ml for killing assays. PMNL viability was assayed by trypan blue exclusion before and after each assay and was consistently > 95%.

Phagocytosis by Direct Counts.

Phagocytosis assays were conducted according to the procedure of Friedman *et al.* (1987) with modification. A bacteria:PMNL ratio of 60:1 was chosen to enhance visualization of the cell-associated bacteria and for statistical analyses. Briefly, *B. pertussis* and *E. coli* were opsonized with NS, IgG fraction, HAPA, complement, or antibody + complement for 20 min in a 37°C water bath. Opsonized bacteria and PMNL were added to 15 ml conical tubes containing 5 ml gel-HBSS. Tubes were incubated at 37°C in an end-over-end rotator. At the end of 60 min, 10% neutral buffered formalin was added to each tube to stop phagocytosis and to fix the PMNL. Tubes were

centrifuged to pellet the PMNL. The pellets were washed with gel-HBSS and resuspended in a few drops of NS. Smears were made on glass slides using a Shandon Cytospin I centrifuge, air dried, methanol fixed, and Giemsa stained. The number of microorganisms associated with 50 PMNL was counted directly by light microscopy. Results were expressed as the ratio of associated bacteria per PMNL.

Phagocytosis by Flow Cytometry.

Flow cytometric analysis of phagocytosis by trypan blue quenching of extracellular fluorescent bacteria was conducted according to the procedure of Hed (1986) with modification. *E. coli* and all three virulent *B. pertussis* strains were incubated individually at a concentration of 10^8 bacteria/ml in 0.1 M carbonate buffer, pH 9.6, containing 0.1 mg/ml fluorescein isothiocyanate (FITC; Sigma, St. Louis, MO) in DMSO (Sigma) for 30 min in a 37°C water bath. The bacteria were washed five times by centrifugation and resuspended at their original concentrations prior to aliquoting and freezing at -20°C. Phagocytosis assays using bacteria:PMNL ratios of 60:1 and 1:20 were conducted as described above. Flow cytometric analysis was performed on the Becton Dickinson FACSTAR analyzer. The fluorescent signal associated with PMNL was amplified by a four decade logarithmic amplifier. Data analysis was performed on a Hewlett Packard 9000 series computer (Model 310) using the Consort 30 version E program. A gate was set using PMNL alone to eliminate collection of events representing dead PMNL, clumps of bacteria, and debris. Ten thousand events per sample were collected to determine the percent PMNL with cell-associated FITC-labelled bacteria. An

equal volume of trypan blue (2 mg/ml in 0.02 M citrate buffer) was added to each sample prior to re-collection. The percent PMNL with intracellular FITC-labelled bacteria was calculated. Flow cytometric analysis was performed by Charles Gschwind and Kathleen Kunke, Arizona Cancer Center, University of Arizona Health Sciences Center.

Killing Assays.

A bacteria:PMNL ratio of 1:20 was chosen to enhance killing of phagocytosed bacteria according to the procedure of Horwitz and Silverstein (1980). Briefly, *E. coli* and *B. pertussis* were opsonized in NS and HAPA, respectively, for 20 min in a 37°C water bath. Each assay was conducted in a final volume of 1 ml in polypropylene tubes. Tubes for 60 or 120 min time points were incubated at 37°C in an end-over-end rotator; tubes for 0 min time points were held on ice. In early experiments, PMNL were sonicated for 20 sec with a micro-tip attached to a sonicator (Heat Systems Ultrasonics, Inc., Plainview, NY) set at position 3. Due to instrument failure, PMNL in later experiments were lysed using sterile deionized water. All tubes were held on ice for 5 min to stop phagocytosis. The contents of each tube was transferred to a sterile microcentrifuge tube and pelleted for 12,000 RPM for 3 min. The supernatants were removed and the pellets were resuspended in 1 ml deionized water. Dilution plate counts were made on LB agar for *E. coli* and on charcoal agar for *B. pertussis.* Results were expressed as the percentage of colony forming units (CFU) per time point relative to the CFU of each organism at 0 min (100%).

Electron Microscopy (EM).

PMNL were allowed to phagocytose *B. pertussis* or *S. aureus* for 60 or 120 min at a ratio of 40 bacteria:1 PMNL. This ratio was used to aid in the ability to observe *B. pertussis* in the EM preparations. Samples were pelleted in a microcentrifuge at 12,000 RPM for 15 sec. The pellets were fixed in 0.1 M cacodylate buffer (pH 7.4) containing 2% glutaraldehyde and 1% sucrose for 60 min at 4°C. Phagosome-lysosome fusion was monitored histochemically by the Gomori technique (1952) as modified by Akporiaye *et al.* (1983) for the presence of the lysosomal enzyme marker acid phosphatase. ß-glycerophosphate (Grade III; Sigma) was used as the enzyme substrate and lead nitrate was used as the capture agent (Gomori, 1952). Fixed cells were washed in cold cacodylate buffer and then incubated in 0.2 M Tris-maleate (pH 5.0) buffered reaction medium for 60 min at 37°C in a gyratory water bath. Pellets were washed after incubation with Tris-maleate buffer and post-fixed in cacodylate buffer containing osmium tetraoxide for 60 min. Specimens were then washed and dehydrated through an alcohol series into propylene oxide. The prepared cells were embedded in Spurr and sectioned. Sections were placed on specimen grids, stained with uranyl acetate and lead citrate, and examined on a Jeol 100 CX2 transmission electron microscope. In these experiments at least 50 PMNL containing bacteria in phagocytic vacuoles were observed for determination of a positive or negative acid phosphatase reaction indicative of phagosome-lysosome fusion and the percentage of fusion was determined.

EM experiments were conducted with the expert assistance of Morey Setareh; Dr. Claire Payne, Dept. of Pathology, University of Arizona Health

Sciences Center; and Dr. Emmanuel Akporiaye, Dept. of Microbiology and Immunology, University of Arizona Health Sciences Center.

Respiratory Burst Activity Determined by Chemiluminescence (CL).

CL assays were performed according to the procedure of Friedman *et al.* (1987) with modification. Briefly, 250 µl of PMNL were added to dark-adapted glass scintillation vials containing 5 ml gel-HBSS and 20 µl of a 27.5 mM solution of luminol (Sigma) in DMSO. Background counts were made at room temperature in a Beckman LS-250 liquid scintillation counter before the addition of *S. aureus* or *B. pertussis* (bacteria to PMNL ratio of 60:1) which had been opsonized in NS or HAPA as described previously. Results were expressed as the average counts per minute (cpm; experimental cpm minus background cpm) and were presented as the percentage of maximum CL relative to the percentage of maximum CL induced by *E. coli* opsonized in NS (control).

Respiratory Burst Activity Determined by Superoxide Anion.

Superoxide anion production assays were performed according to the procedure of Akporiaye *et al.* (1990). Briefly, 250 µl of PMNL in Dulbecco's PBS with 5 mM glucose and 0.1% gelatin (PBSg) were added to tubes containing 80 µmol/l ferricytochrome C (horse heart type VI; Sigma catalog no. C-7752) and opsonized *E. coli*, *S. aureus*, or *B. pertussis* (60 bacteria: 1 PMNL ratio) in a final reaction volume of 2.1 ml. Bacteria were opsonized as described previously or by incubation for 60 min in a shaking 37°C water bath,

microcentrifuged for 3 min, washed, and resuspended to the original volume in PBSg. Phorbol myristate acetate (PMA; Sigma; 100 ng/ml) was used as a positive control. In some experiments, 100 µg/ml superoxide dismutase (SOD; from bovine erythrocytes; Sigma) was added to determine the amount of SOD-inhibitable superoxide produced. Tubes were incubated for 30 or 60 min in a slowly shaking 37°C water bath. After incubation, the tubes were centrifuged at 3000 RPM for 10 min. The supernatants were transferred to clean tubes. The absorbance of the supernatants was measured at 550 nm compared to a control tube containing only cytochrome C in PBSg. Change in absorbance was determined by subtracting the sum of the control tubes from the sum of the experimental tubes and dividing by 2 to account for the duplicates. The amount of cytochrome C reduced was used as an indicator of superoxide formation. The amount of superoxide formed was calculated using the extinction coefficient of 2.1×10^4 $M^{-1}cm^{-1}$ at 550 nm. Results are given as nmol superoxide produced/5×10^5 PMNL/60 min. Superoxide anion experiments were conducted with the expert assistance of Dr. Emmanuel Akporiaye, Dept. of Microbiology and Immunology, University of Arizona Health Sciences Center.

Respiratory Burst Activity Determined by Flow Cytometry.

Flow cytometric analysis of reactive oxygen molecules was conducted according to the procedure of Bass *et al.* (1983) with modification. Briefly, PMNL were harvested as described above and resuspended in PBSg. PMNL were loaded with a final concentration of 5 µM dichlorofluorescin diacetate (DCFH-DA; Eastman Kodak, Rochester, NY) for 15 min in a quickly shaking

37°C water bath. After the loading period, 20 µl each of 0.25 mM EDTA and 0.25 mM sodium azide were added per ml of PMNL. Background fluorescence (fluorescence of unstimulated PMNL) was determined on a Becton Dickinson FACScan analyzer. DCFH-DA loaded PMNL were added to NS-opsonized *E. coli*, HAPA- or IgG fraction-opsonized virulent *B. pertussis*, or 100 ng PMA/ml. The bacteria:PMNL ratio used was 60:1. Tubes were set up in duplicate for six 10-min time points with final volumes of 2 ml. The tubes were incubated in a quickly shaking 37°C water bath. At each time point, the reactions were stopped by the addition of ice cold PBSg and analyzed. FACScan analysis was conducted with the same parameters as previously described for the FACSTAR analysis of phagocytosis. Results were presented as the average maximum fluorescence intensity (FI) associated with the stimulated PMNL (experimental maximum FI minus background maximum FI).

Serum bactericidal activity against *B. pertussis*.

Serum killing assays were conducted in a manner similar to PMNL killing assays except that additional gel-HBSS replaced PMNL in each assay. Briefly, for each assay 2.5×10^5 virulent *B. pertussis* were opsonized in NS as described previously. The volume was increased to 1 ml with gel-HBSS, incubated in an end-over-end rotator for 60 min, and dilution plate counts made on charcoal agar. In order to determine the importance of the classical complement pathway in bactericidal activity, 10 mM or 15 mM EGTA was added to NS, gel-HBSS, and dilution blanks to chelate calcium and block complement activation.

Statistical Analyses.

The significance of differences between results was calculated by analysis of variance (ANOVA). All significant differences between groups identified by two-way ANOVAs at the 95% confidence level or greater was confirmed by post-hoc testing. Statistical analyses was performed by the Office of Biostatistical Services, University of Arizona Health Sciences Center.

TABLE 2-1. *B. pertussis* (BP) Strains Used in This Study

Strain	Phenotype	Source/ Reference
BP165	Virulent	FDA
BP504	Virulent	FDA
BP338	Virulent derivative of Tohama I	Weiss, 1983
BP326	Spontaneous avirulent derivative of Tohama III	Weiss, 1983
BP347	Avirulent Tn5 mutant of BP338	Weiss, 1983
BP348	BPAC$^-$Hly$^-$ Tn5 mutant of BP338	Weiss, 1983
BP349	BPAC±Hly$^-$ Tn5 mutant of BP338	Weiss, 1983
BP352	BPAC$^+$Hly$^-$ Tn5 mutant of BP338	Weiss, 1983
BP353	FHA$^-$ Tn5 mutant of BP338	Weiss, 1983
BP357	PT$^-$ Tn5 mutant of BP338	Weiss, 1983
BP325	Spontaneous FHA$^-$ variant of Tohama I	Zhang, 1985
BP537	Spontaneous avirulent derivative of BP356 (a variant of BP338)	Relman, 1989
BP101	FHA$^-$ deletion mutant of BP356	Relman, 1989
BPTox 6	PT$^-$ deletion mutant of BP356	Relman, 1989
BP101/ Tox6	FHA$^-$PT$^-$ deletion mutant of BP356	Relman, 1990

CHAPTER III

RESULTS

PART I: CHARACTERIZATION OF NORMAL HUMAN SERA (NS) AND HUMAN ANTI-*B. PERTUSSIS* ANTIBODY (HAPA).

NS and HAPA were analyzed for complement levels by rate nephelometry and for anti-*B. pertussis* antibody titers by ELISA (Winston, 1987). NS used in these studies had normal complement levels, while HAPA had no demonstrable complement level. Anti-whole cell *B. pertussis* IgG titers of NS were within a range of 1:200 to 1:640 (mean 1:400) compared to a 1:300 titer for HAPA. Both NS and HAPA had anti-whole cell *B. pertussis* IgA and IgM titers of < 1. Anti-whole cell *B. pertussis* titers of NS and of HAPA against each of three virulent strains were not significantly different. All NS alone killed over 98% of all three virulent *B. pertussis* strains in less than 20 min at 37°C. HAPA did not significantly reduce the number of CFU even after 120 min at 37°C (data not shown).

An IgG fraction of NS pooled from four high titer individuals was made to substantiate the results of the commercially prepared HAPA. When diluted to the same 1 mg protein/ml concentration present in HAPA, this IgG fraction had similar anti-whole cell *B. pertussis* titers as determined by ELISA which were not significantly different for the three virulent strains. Because the fractionation procedure eliminates complement, complement levels were not determined.

Serum Bactericidal Activity Against *B. pertussis.*

Addition of 10 mM EGTA to NS has been shown to specifically inhibit the classical complement pathway. While NS alone completely killed all three *B. pertussis* strains within 30 min, the addition of 10 mM EGTA completely eliminated serum killing (Fig. 3-1). This indicates that the classical complement pathway is of greater relevance than the alternate pathway in host defense against *B. pertussis.* HAPA alone had no effect on viability of these bacteria, again supporting the importance of complement in this type of bactericidal activity.

PART II: INTERACTIONS BETWEEN VIRULENT *B.PERTUSSIS* STRAINS AND PMNL

Analysis of PMNL Phagocytosis of *B. pertussis* by Direct Counts.

In initial experiments, *B. pertussis* (BP338) and *S. aureus* were opsonized with NS or HAPA and phagocytosis was monitored by light microscopy to determine which opsonin induced optimal PMNL phagocytosis (Fig. 3-2). Maximal phagocytosis of BP338 required opsonization with HAPA (Fig. 3-2D), while NS (Fig. 3-2C) or heat-inactivated NS (data not shown) produced negligible phagocytosis. NS provided the optimal opsonin for *S. aureus* with HAPA inducing minimal phagocytosis (Fig. 3-2A and 3-2B). This is not surprising since complement is required for phagocytosis of *S. aureus* (Verhoef, 1977) and is not present in HAPA.

In later experiments, the three virulent *B. pertussis* strains and *E. coli* were opsonized with NS or HAPA or IgG fraction to monitor phagocytosis. Internalization of cell-associated bacteria (i.e., phagocytosis) was confirmed

concurrently by flow cytometry (see below). Figure 3-3 shows negligible uptake of all three virulent *B. pertussis* strains opsonized in NS compared to an average of 14 *E. coli* per PMNL. For all three strains there was a significant increase in phagocytosis using HAPA or IgG fraction as opsonins ($P < 0.01$) compared to NS as an opsonin. Except for BP338, there was no significant difference between phagocytosis induced by HAPA and that induced by IgG fraction. Phagocytosis of all three strains opsonized in 5% guinea pig complement alone was negligible. Therefore, PMNL phagocytosis of *B. pertussis* appears to require opsonization with antibody, rather than complement. Addition of 5% guinea pig complement to HAPA or IgG fraction (original antibody concentration maintained) prior to opsonization altered phagocytosis of the *B. pertussis* virulent strains. BP338 opsonized in HAPA alone was better phagocytosed than when opsonized in HAPA + complement ($P < 0.01$). On the other hand, phagocytosis of both BP504 and BP165 was increased by opsonization in HAPA + complement ($P < 0.01$). Opsonization with IgG fraction + complement induced greater phagocytosis of all three *B. pertussis* strains than IgG fraction alone ($P < 0.05$ for BP504 and $P < 0.01$ for BP165). The decrease in phagocytosis of BP338 opsonized in HAPA + complement may be due to strain variation or may be an anomaly since IgG fraction + complement increased phagocytosis of all three *B. pertussis* strains. Phagocytosis of *E. coli* opsonized in HAPA or IgG fraction, with or without complement, or complement alone was negligible. This is not surprising since complement and IgM are required for phagocytosis of *E. coli* (Horwitz, 1980).

In order to determine if phagocytosis is influence by the viability of the bacteria, fluorescein isothiocyanate (FITC)-labelled bacteria were opsonized

in NS, HAPA, or IgG fraction. The FITC-labelling procedure killed > 98% of all bacterial strains (see Methods and Materials). Phagocytosis of all three FITC-labelled *B. pertussis* strains opsonized in NS was negligible. Although there were 9 NS-opsonized, FITC-labelled *E. coli* per PMNL, this was significantly different from the 14 live *E. coli* per PMNL ($P < 0.05$). Phagocytosis of live vs. FITC-labelled *B. pertussis* strains opsonized in HAPA was 26 vs. 21 BP338 per PMNL, 10 vs. 12 BP504 per PMNL, and 11 vs. 11 BP165 per PMNL. Phagocytosis of live vs. FITC-labelled *B. pertussis* strains opsonized in IgG was 12 vs. 15 BP338 per PMNL, 10 vs. 13 BP504, and 13 vs. 13 BP165 per PMNL. Since there were no significant differences at the $P < 0.01$ level between live and dead bacteria of the same strain in any serum tested, these results indicate that PMNL phagocytose live and dead *B. pertussis* equally well.

Analysis of PMNL Phagocytosis of *B. pertussis* by Flow Cytometry.

Direct counts of Giemsa stained smears cannot distinguish between those bacteria which are adherent to the surface of PMNL and those bacteria which are internalized. A flow cytometry fluorescence quenching methods using FITC-labelled bacteria (Hed, 1986) can differentiate between attached and ingested bacteria associated with phagocytes. It has been used to monitor phagocytosis of *Candida albicans* (Hed, 1986; Bjerknes, 1984), red blood cells (Loike, 1983), *E. coli* (Ohman, 1982), and *S. aureus* (Bassoe, 1984). The fluorescence associated with PMNL is derived from the fluorescence of the total number of FITC-labelled bacteria--both adhered and internalized--associated with those PMNL. The addition of trypan blue

chemically quenches the fluorescence of all extracellular, but not intracellular, bacteria. In this case the fluorescence associated with PMNL is derived only from the fluorescence of intracellular bacteria.

Fluorescence quenching was performed concurrently with direct phagocytosis studies. Bacteria-to-PMNL ratios of 60:1 (the phagocytosis assay ratio) and 1:20 (the killing assay ratio) were examined. FITC-labelled *E. coli* was opsonized with NS and all three FITC-labelled *B. pertussis* strains were opsonized with HAPA. Figure 3-4 shows representative fluorescence histograms with (intracellular bacteria only) and without (all cell-associated bacteria) the addition of trypan blue for E. coli and for BP338 at these ratios. Similar histograms were obtained for BP504 and BP165 (data not shown). Because the FACSTAR cannot register the small size of individual *E. coli* and *B. pertussis*, one cannot determine the average number of bacteria per PMNL to substantiate our direct count values. However, at both ratios over 90% of PMNL determined to have associated *E. coli* or *B. pertussis* had phagocytosed those bacteria and less than 10% of the PMNL still had any non-phagocytosed bacteria remaining on their surfaces. The flow cytometry values indicate that the majority of PMNL-associated bacteria were indeed internalized. This confirms that the direct count values are a direct indicator of PMNL phagocytosis.

Survival of *B. pertussis* After PMNL Phagocytosis.

Once it was established that HAPA induced the highest level of *B. pertussis* uptake, the efficiency of PMNL killing of phagocytosed *B. pertussis* was ascertained (Table 3-1). At 60 min, 79% BP165 survived

PMNL bactericidal activity while 97% BP504 and 108% BP338 survived. In control experiments, 1% *E. coli* survived showing the normal bactericidal function of PMNL. At 120 min, survival of virulent *B. pertussis* strains was less extensive, although still significantly more than that of control *E. coli*, whose survival was unchanged (Table 3-1). The *B. pertussis* strains varied in their resistance to PMNL killing with BP338 > BP504 > BP165. The difference in survival of *E. coli* controls compared to that of all three *B. pertussis* strains was highly significant at 60 as well as at 120 min ($P < 0.001$). The results demonstrate that virulent *B. pertussis* strains are not readily killed and that these organisms can survive intracellularly within PMNL after phagocytosis. In addition, they indicate strain variation in their ability to survive PMNL bactericidal activities after phagocytosis.

Because virulent *B. pertussis* survive PMNL phagocytosis and killing there must be one or more mechanisms which allow these bacteria to survive. Electron microscopy was used to monitor phagosome-lysosome fusion and several techniques were used to monitor respiratory burst activity.

Electron Microscopy (EM) of PMNL Interactions With *B. pertussis*.

EM was used to monitor phagosome-lysosome fusion by histochemical staining for the presence of the lysosomal enzyme marker acid phosphatase. Primary lysosomes contain acid phosphatase which is enzymatically active in the pH 4-5 environment created by the fusion of lysosomes with phagosomes. A reaction of the active enzyme with its substrate, ß-glycerophosphate, and lead nitrate causes precipitation of an electron-dense reaction product, lead phosphate. Thus, if phagosome-lysosome fusion is normal, phagocytic

vacuoles containing bacteria will also show the presence of this positive acid phosphatase staining (Akporiaye, 1983).

Electron micrographs of most phagocytosed bacteria typically demonstrate large phagocytic vacuoles enclosing the bacteria (Armstrong, 1981; Hart, 1972). Phagosome-lysosome fusion is indicated by substantial electron-dense lead phosphate reaction product around and within the vacuoles. Bacteria can often be observed to be undergoing degradation and destruction (Akporiaye, 1983; Armstrong, 1981; Hart, 1972; Hart, 1987; Horwitz, 1983; Lowrie, 1979; Rikihisa, 1980; Wyrick, 1978). Figure 3-5 shows control EM experiments with *S. aureus* demonstrating typical phagosome-lysosome fusion occuring with the bacteria enclosed in large phagocytic vacuoles with substantial electron-dense lead phosphate reaction product (Fig.3-5A). In some of the phagocytic vacuoles, *S. aureus* appears to be undergoing degradation and destruction. Figure 3-5 also illustrates acid phosphatase-stained lysosomes which have not fused with phagosomes containing *S. aureus.*

Electron micrographs with BP338 were dramatically different. Figures 3-6A and 6C show BP338 contained in tightly fitting phagocytic vacuoles in the cytoplasm of PMNL 60 min after phagocytosis. No positive acid phosphatase histochemical staining was observed in association with vacuoles containing *B. pertussis.* A large number of acid phosphatase stained lysosomes are present throughout the PMNL cytoplasm. Many lysosomes are in close proximity to vacuoles containing *B. pertussis,* but no fusion has occured. Low levels of bacterial acid phosphatase can be observed in the membrane of *B. pertussis* in Figure 3-6D, which is distinct

from the electron-dense acid phosphatase reaction observed in vacuoles in close proximity to phagosomes containing *B. pertussis*.

Quantitation of PMNL phagosome-lysosome fusion was done following phagocytosis of *S. aureus* and *B. pertussis* at 60 and 120 min. After 60 min of phagocytosis, 60% of vacuoles containing *S. aureus* were positive for acid phosphatase staining. This percentage increased to 66% by 120 min. After both 60 and 120 min of phagocytosis only 20% and 21%, respectively, of *B. pertussis*-containing phagocytic vacuoles were positive for acid phosphatase staining. These results demonstrate that *B. pertussis* inhibits phagosome-lysosome fusion.

Analysis of PMNL Respiratory Burst Activity Induced by *B. pertussis* Via Chemiluminescence (CL) Assay.

CL occurs during the generation of superoxide and other molecules of the PMNL respiratory burst (Briheim, 1984). This phenomenon can be amplified by the use of luminol in a CL assay (Briheim, 1984; Friedman, 1987). Initial studies employing NS opsonization showed a greatly decreased CL response induced by BP338 when compared to control *S. aureus* suggesting inhibition of CL by *B. pertussis* (Fig. 3-7). However, this decreased CL response was due to poor phagocytosis of BP338 by PMNL rather than a bacterially induced inhibition (Fig. 3-3). In this context, lack of CL response to *B. pertussis* is understandable. HAPA-opsonized BP338 induced a CL response comparable to *S. aureus* (Fig.3-7).

CL response of PMNL to all NS-opsonized *B. pertussis* strains tested was 13% or less of control values and in some cases was essentially

nonexistent (Fig. 3-8). CL response when *B. pertussis* was opsonized with HAPA was dramatically different with BP338 most closely attaining control CL levels (96.2%). The CL response induced by BP338 was not significantly different from control levels. BP504 and BP165 induced lower CL response levels than control levels.

Analysis of PMNL Respiratory Burst Activity Induced by *B. pertussis* Via Superoxide Anion Assay.

During the respiratory burst NADPH oxidase becomes activated and reduces molecular oxygen (O_2) to superoxide (O_2^-). Superoxide, a very unstable ion, spontaneously dismutates to hydrogen peroxide and molecular oxygen (Babior, 1984). PMNL superoxide production was monitored by reduction of ferricytochrome C. Figure 3-9 shows the amount of superoxide production induced by *E. coli*, *S. aureus*, and *B. pertussis* (BP338) opsonized by their optimum opsonin compared with the superoxide production of unstimulated PMNL from four individuals. Unstimulated PMNL produced 9.4 ± 12.4 nmol superoxide, indicating significant differences in the responses of individual donor PMNL. Both *E. coli* and *S. aureus* stimulated superoxide production exceeding background levels with means of 26.8 ± 19.0 and 17.8 ± 14.4 nmol superoxide produced, respectively. BP338 was unable to stimulate background levels of superoxide assay with -2 ± 8.3 nmol superoxide produced. Normal PMNL superoxide production was indicated by the production of 548.9 ± 71.8 nmol superoxide in response to PMA. Superoxide production stimulated by all three microbes and PMA was

inhibited over 95% by the addition of exogenous superoxide dismutase (SOD) (data not shown).

Because excess IgG alone can induce PMNL superoxide production, the three bacterial stimuli were washed after opsonization to remove excess free antibody. Washed *E. coli* and *S. aureus* induced less superoxide production than their unwashed counterparts with means of 8.6 ± 4.2 and 1.2 ± 3.0 nmol superoxide, respectively. Superoxide production induced by washed BP338 was slightly higher than that induced by unwashed BP338 with a mean of 0.5 ± 3.5 nmol superoxide. These data suggest that BP338 suppresses PMNL superoxide production, in contrast with the results of CL assays and flow cytometry assays.

Analysis of PMNL Respiratory Burst Activity Induced by *B. pertussis* Via Flow Cytometry.

The respiratory burst of phagocytic cells can be monitored by a variety of methods including CL, oxygen consumption, hexose monophosphate shunt activity, generation of hydrogen peroxide, superoxide, and hydroxyl radicals, or reduction of tetrazolium dye. However, these assays assume that the phagocyte population is homogeneous (i.e., that all cells in the population will respond identically) and that the stimulus will stimulate all cells uniformly. In addition, these assays measure the amount of respiratory burst activity extracellularly, hence indirectly. Bass et al. (1983), developed a technique using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a substrate for toxic oxygen products which can directly monitor the respiratory burst from within phagocytes. DCFH-DA diffuses across the membrane where it is deacetylated

to 2',7'-dichlorofluorescin (DCFH), a nonfluorescent, nondiffusable, highly stable compound. In the presence of hydrogen peroxide DCFH is oxidized to 2',7'-dichlorofluorescein (DCF). DCF is a highly fluorescent, nondiffusable compound which causes phagocytes to fluoresce. Flow cytometry is used to measure the resulting fluorescence of individual phagocytes. This technique has been used to study PMNL phagocytosis of *S. aureus*, *E. coli*, and *Candida albicans* (Szeda, 1984; Bjerknes, 1989; Gordon, 1989; Jacobs, 1989; Rothe, 1990).

Bacteria-to-PMNL ratios of 60:1 and nonspecific stimulation by PMA (100 ng/ml) were examined. *E. coli* was opsonized in autologous NS and all three *B. pertussis* strains were opsonized in HAPA or IgG fraction. Figure 3-10 shows representative fluorescence histograms for *E. coli*, *B. pertussis*, and PMA compared with unstimulated PMNL. Similar histograms were obtained for BP504 and BP165 (data not shown). Respiratory burst activity induced by *B. pertussis* was equivalent to that induced by PMA and was much greater than that induced by *E. coli*. Figure 3-11 shows the mean maximum fluorescent intensity induced by *E. coli*, all three *B. pertussis* strains in both opsonins, and PMA. These figures indicated that the respiratory burst is quite active in PMNL that have phagocytosed *B. pertussis*. This is quite unexpected because purified BPAC has been shown by several studies to completely inhibit phagocytic cell respiratory burst activity (Confer, 1982; Friedman, 1987; Galgiani, 1988).

PART III: INTERACTIONS BETWEEN *B. PERTUSSIS* MUTANTS AND PMNL

Studies were done in order to assess the impact of specific virulence factors on PMNL phagocytosis and killing of *B. pertussis*. These studies used transposon Tn5-generated and deletion mutants (see Table 2-1) in comparison with BP338, the parent strain of most of the mutants.

Analysis of PMNL Phagocytosis of *B. pertussis* Mutants by Direct Counts.

A series of Tn5 mutants created by Weiss *et al.* (1983) and several deletion mutants developed by Relman *et al.* (1989, 1990) were opsonized with NS or HAPA (Fig. 3-12). As seen with the virulent *B. pertussis* strains, the uptake of all *B. pertussis* mutants opsonized in autologous NS was negligible. HAPA induced significantly higher levels of uptake of all mutants except avirulent strains BP347 and BP326 and Tn5 FHA$^-$ mutant BP353. Apparently, the avirulent strains do not bear surface antigens recognizable by HAPA which would allow phagocytosis of these strains to occur. Interestingly, spontaneous FHA$^-$ strain BP325 was more readily phagocytosed than either BP504 or BP165 or any of the other mutants, including BP101 (deletion FHA$^-$ mutant). In contrast, Tn5 FHA$^-$ mutant BP353 responded more like the two avirulent strains than BP325 or BP101. The lack of PT does not appear to inhibit phagocytosis of PT$^-$ mutants BP357 and BPTox 6. This is not particularly surprising since PT$^-$ mutants do produce FHA and other surface molecule to which HAPA might bind. The importance of BPAC in induction of phagocytosis is suggested by the increase in uptake of mutants bearing BPAC, BP349 (BPAC$\pm$Hly$^-$) and BP352 (BPAC$^+$Hly$^-$), compared to BP348

(BPAC$^-$Hly$^-$). However, the same strain variation seen with phagocytosis of the three virulent *B. pertussis* strains can be seen with different mutations of the same virulence factor.

Since neither NS- nor HAPA-opsonized avirulent *B. pertussis* were phagocytosed by PMNL, one of the avirulent mutants was used to produce rabbit antiserum. Opsonization with rabbit anti-avirulent antiserum (RAAA) was effective in increasing phagocytosis of avirulent bacteria to at least 10 bacteria per PMNL. This strongly suggests that opsonic antibody can be made against less immunogenic surface antigens than FHA, PT, or BPAC.

Survival of *B. pertussis* Mutants After PMNL Phagocytosis.

Once the optimum parameters for phagocytosis of the mutants was determined, the efficiency of PMNL killing of phagocytosed *B. pertussis* mutants was ascertained (Table 3-2). The avirulent mutants were opsonized in RAAA; the other mutants were opsonized in HAPA. Interestingly, 65% of FHA$^-$ deletion mutant BP101 survived 60 min of PMNL bactericidal activity compared to 97% survival of spontaneous FHA$^-$ mutant BP325, indicating strain variability. Survival of double mutant BP101/Tox6 was 59%. It is difficult to determine the role of FHA in survival of this double mutant because of the difference in survival of the two FHA$^-$ mutants. It is possible that the decreased survivability of BP101/Tox6 is due to the loss of PT since 57% of BPTox6 (PT$^-$) survived. Seventy seven per cent of BP348 (BPAC$^-$Hly$^-$) survived PMNL bactericidal activity, suggesting that the loss of BPAC is not as deleterious to ability to survive intracellularly as the loss of PT or possibly FHA. Survival of avirulent mutants BP537 and BP347 were 76% and 75%, respectively.

Unfortunately, the survival of the avirulent mutants cannot be compared directly with the survival of the other mutants because of the difference in opsonins. However, all these data indicate that there is an inherent ability of *B. pertussis* to survive intracellularly within PMNL after 60 min. In control experiments, less than 1% of *E. coli* survived indicating the normal bactericidal function of PMNL.

Overall, mutations in the PT, BPAC, and FHA genes did decrease intracellular survival of *B. pertussis* in PMNL. Yet avirulent mutants demonstrated comparable decreased survival. These results suggest that *bvg*-regulated gene products play some role in survival of *B. pertussis* within PMNL, but that other, unknown, non-*bvg*-regulated gene products also appear to be important in this process.

Analysis of PMNL Respiratory Burst Activity Induced by *B. pertussis* Via Chemiluminescence (CL).

As seen with the virulent *B. pertussis* strains, CL response appeared inhibited when the mutants tested were opsonized with NS (Fig.3-8). However, this decreased CL response was due to poor phagocytosis of the mutants (Fig.3-12). Avirulent strains BP326 and BP347 opsonized with HAPA induced 15% and 25% of control CL response, respectively. This was particularly interesting since HAPA induced negligible levels of phagocytosis (Fig.3-12). Assays with RAAA-opsonized avirulent bacteria were not performed due to insufficient RAAA. BP348 (BPAC$^-$Hly$^-$) opsonized in HAPA induced a CL response similar to that induced by BP326. This was surprising

since BP348 survives PMNL bactericidal activity and the virulent *B. pertussis* strains do induce a very active respiratory burst (Fig.3-8 and 3-10).

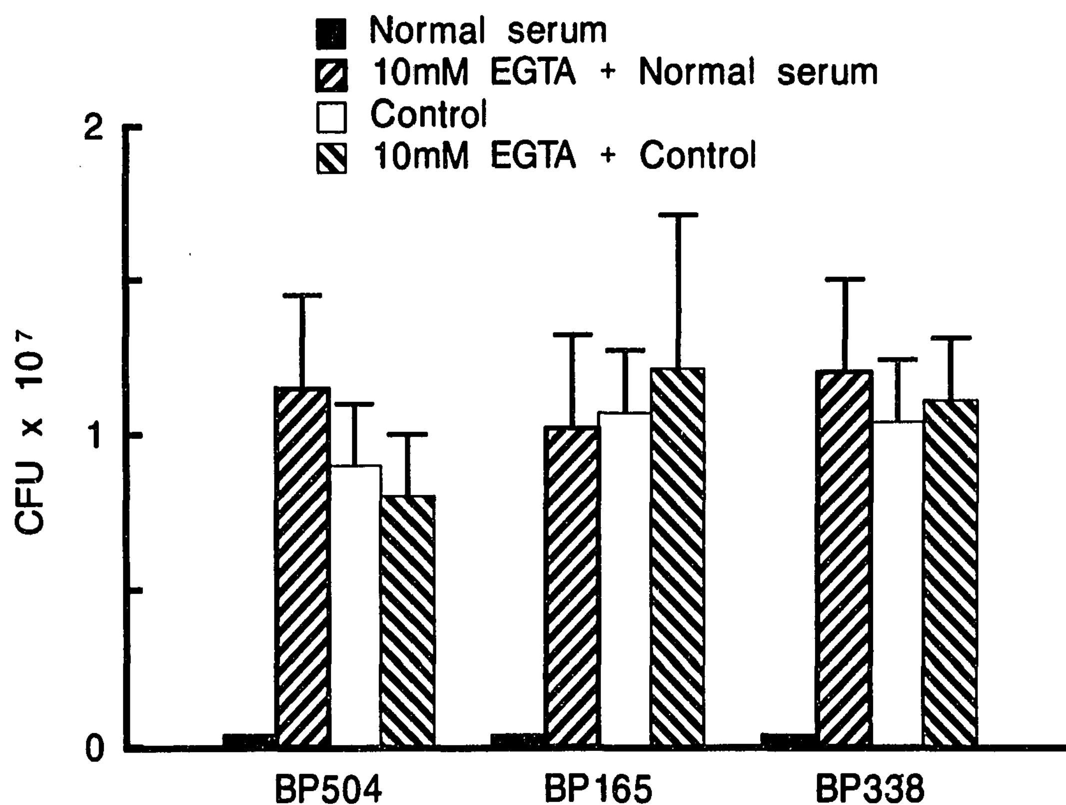

Fig. 3-1. Serum bactericidal activity against virulent *B. pertussis* strains is inhibited by blockade of the classical complement pathway. Opsonized bacteria were incubated in gel-HBSS for 60 min and dilution plate counts made to determine survival. Addition of 10 mM EGTA eliminated serum killing without adversely altering survival itself. Values represent the mean ± SD of 3 duplicated experiments.

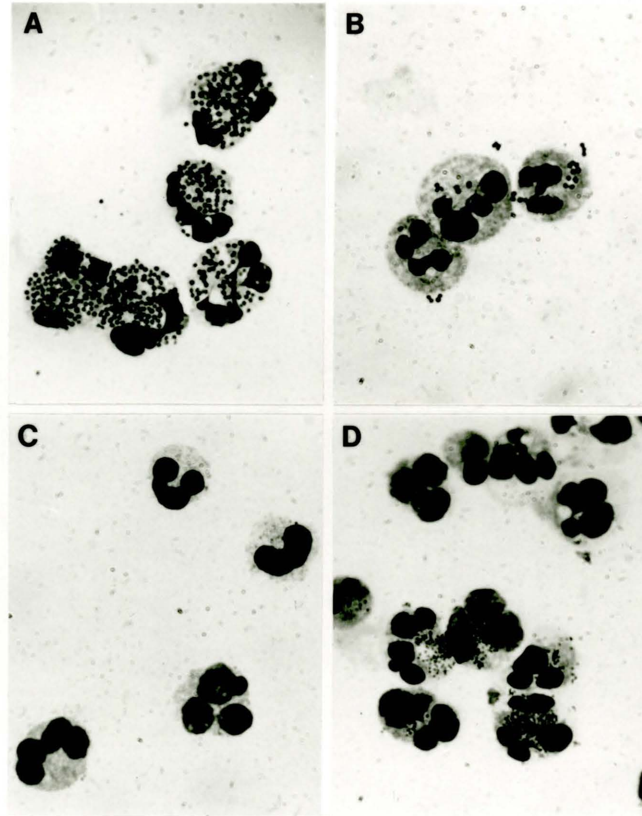

Fig. 3-2. Phagocytosis of *S. aureus* and BP338 opsonized with NS or HAPA. Opsonized BP338 and *S. aureus* were incubated with 2 x 10^6 PMNL/ml in a 60:1 ratio for 60 min. Smears were made using a Cytospin centrifuge, Giemsa stained, and examined by light microscopy. PMNL phagocytosis of *S. aureus* was optimized when opsonized with NS (A) rather than HAPA (B). PMNL phagocytosis of BP338 was optimized when opsonized with HAPA (D) rather than NS (C).

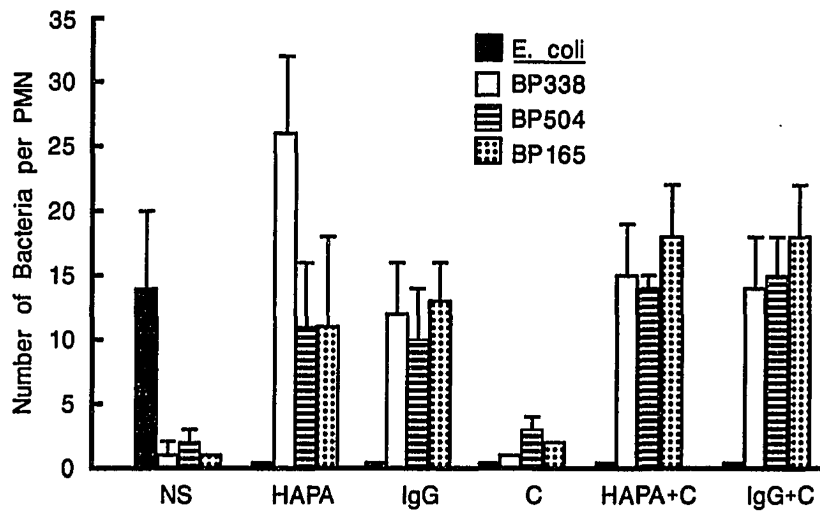

Fig. 3-3. PMNL phagocytosis of *E. coli* and *B. pertussis* opsonized in autologous NS, HAPA, IgG fraction, complement (C), and antibody + complement. Values presented are the numbers of PMNL-associated bacteria per PMNL determined by direct counts of Giemsa stained cytospin smears. Values represent the mean ± SD of at least 3 experiments done in duplicate.

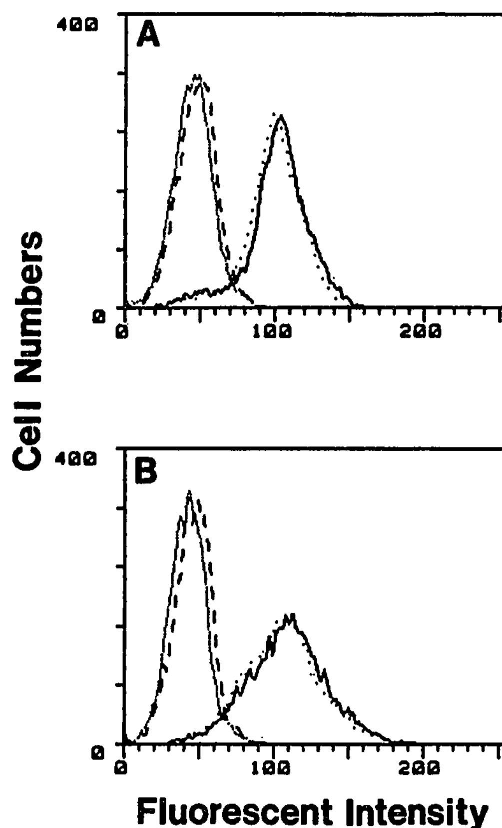

Fig. 3-4. Flow cytometric analysis of FITC-labelled *E. coli* (A) opsonized in NS and BP338 (B) opsonized in HAPA. Opsonized bacteria were incubated with PMNL in a 60:1 or a 1:20 ratio for 60 min. Ten thousand events per sample were collected with and without addition of an equal volume of trypan blue. The fluorescence histograms are representative of at least 3 experiments. Solid lines = 60:1 ratios without trypan blue; widely spaced dotted lines = 60:1 ratios with trypan blue; closely dotted lines = 1:20 ratios without trypan blue; dashed lines = 1:20 ratios with trypan blue.

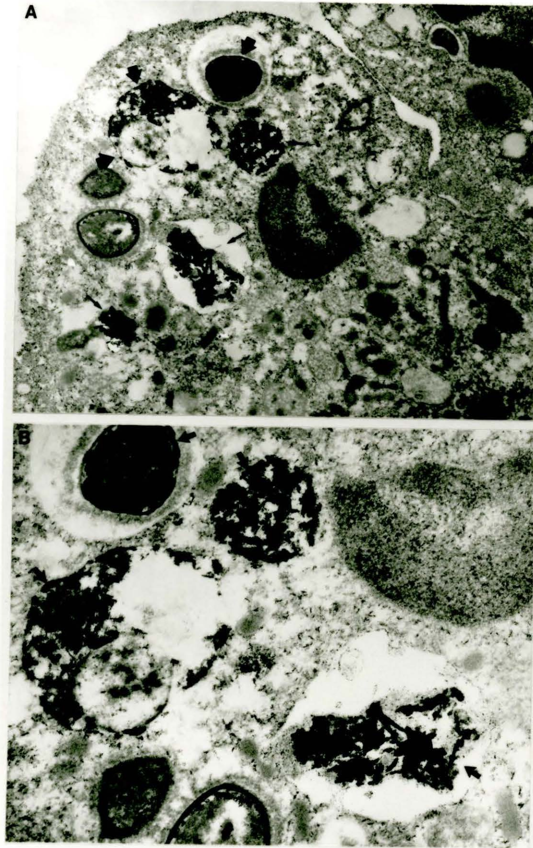

Fig. 3-5. Electron micrographs of normal phagosome-lysosome fusion after PMNL phagocytosis of *S. aureus* opsonized in NS. (A) *S. aureus* present in large phagosome-lysosomes (large arrows) demonstrating a positive acid phosphatase staining reaction seen as an abundant electron-dense lead phosphate reaction product (16,500x). Acid phosphatase positive lysosomes not fused with phagosomes containing *S. aureus* can also be observed (small arrows). (B) Higher magnification of (A) showing the strongly positive acid phosphatase staining reaction (arrows) (38,700x).

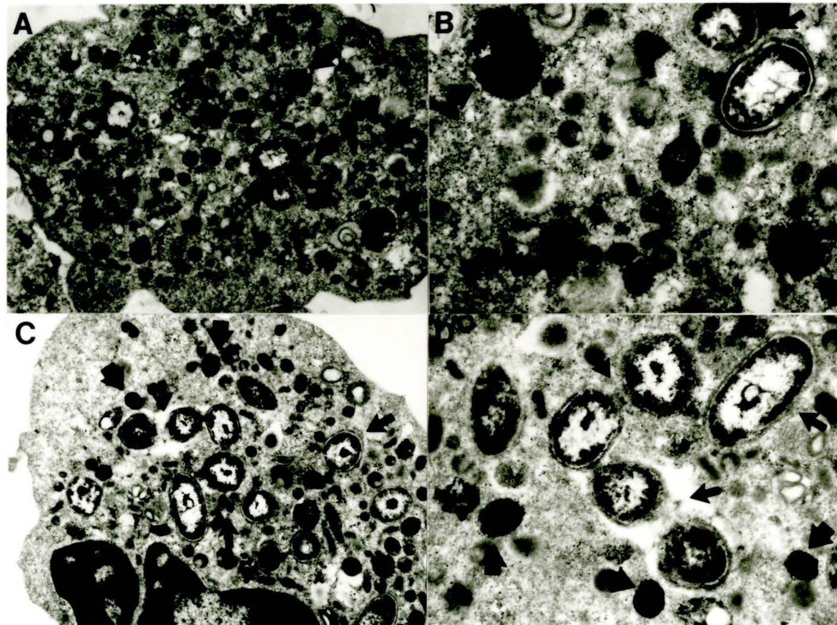

Fig. 3-6. Electron micrographs of inhibition of phagosome-lysosome fusion after PMNL phagocytosis of *B. pertussis* (BP338) opsonized in HAPA. (A and C) After 60 min phagocytosis PMNL contained BP338 in tightly fitting vacuoles demonstrating negative acid phosphatase staining reaction (small arrows) (18,400x). No acid phosphatase staining can be observed in association with vacuoles containing *B. pertussis*. Acid phosphatase positive lysosomes can also be observed throughout the PMNL cytoplasm (large arrows). (B) Higher magnification of (A) (38,300x). Many lysosomes (arrowheads) are near vacuoles containing *B. pertussis* (arrows), but no fusion has occurred. (D) Higher magnification of an area of (C) demonstrating low levels of bacterial acid phosphatase in the *B. pertussis* membrane, which is distinct from the electron-dense acid phosphatase reaction seen in the lysosomes.

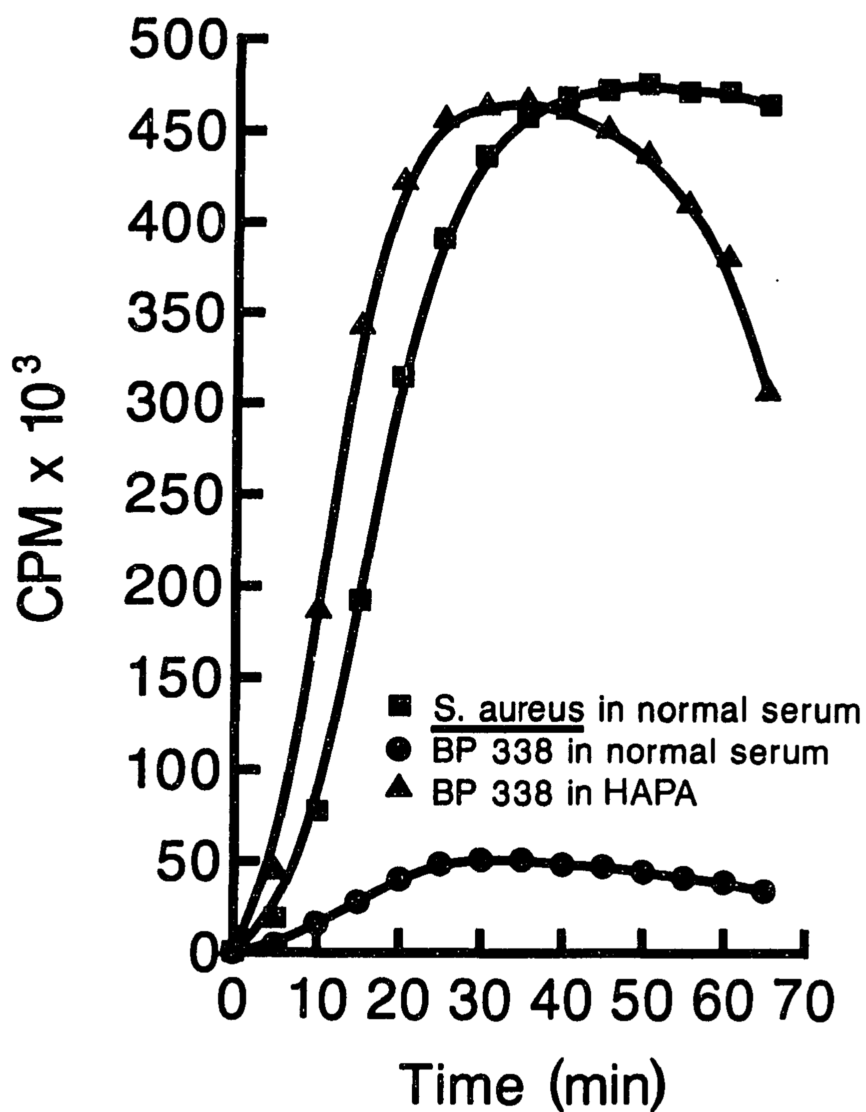

Fig. 3-7. Effect of *B. pertussis* on PMNL CL response. PMNL were incubated with opsonized BP338 or *S. aureus* in a 60 bacteria:1 PMNL ratio and assayed for CL response. Initial results suggest inhibition of PMNL CL induced by *B. pertussis* compared with that induced by *S. aureus*. Such inhibition could be overcome by opsonization of BP338 in HAPA. Values represent the mean of duplicate samples of a representative experiment.

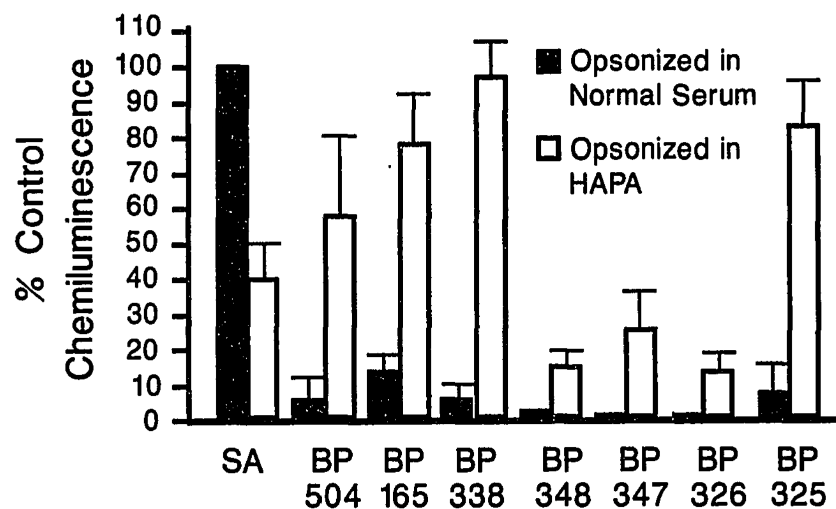

Fig. 3-8. Induction of PMNL CL by *B. pertussis* strains and *S. aureus* opsonized with NS or HAPA. CL response was assayed as described previously. CL response is given as the maximum CL intensity observed compared with control (*S. aureus* opsonized in NS). Values represent the mean ± SD of at least 3 duplicated experiments.

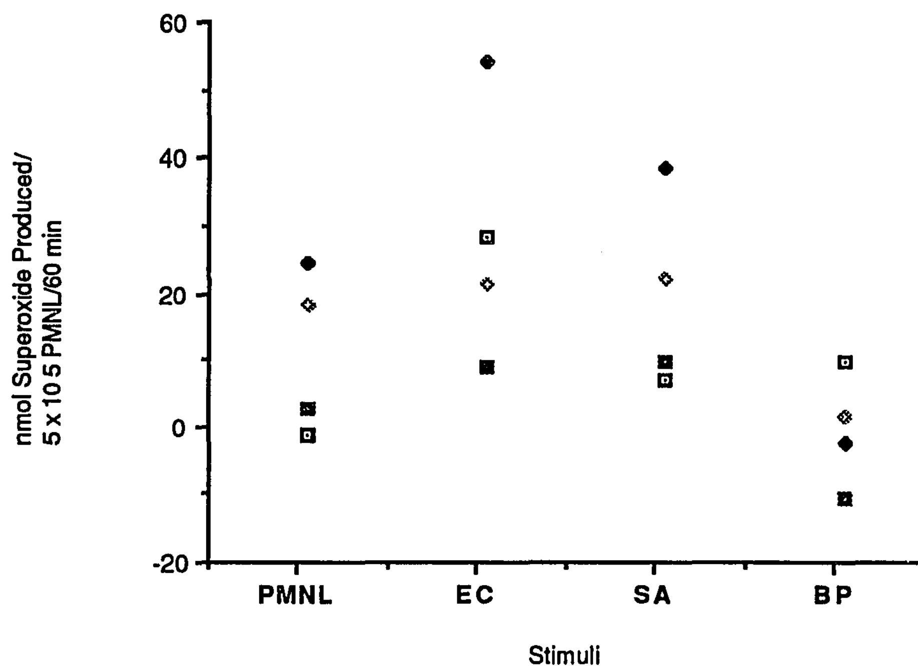

Fig. 3-9. Induction of PMNL superoxide production by *E. coli*, *S. aureus*, and *B. pertussis* (BP338). *E. coli* and *S. aureus* were opsonized in NS; BP338 was opsonized in HAPA. Superoxide production was monitored by ferricytochrome C reduction. Values represent the nmol superoxide produced in four individual experiments. Horizontal bar represents the mean of the experiments.

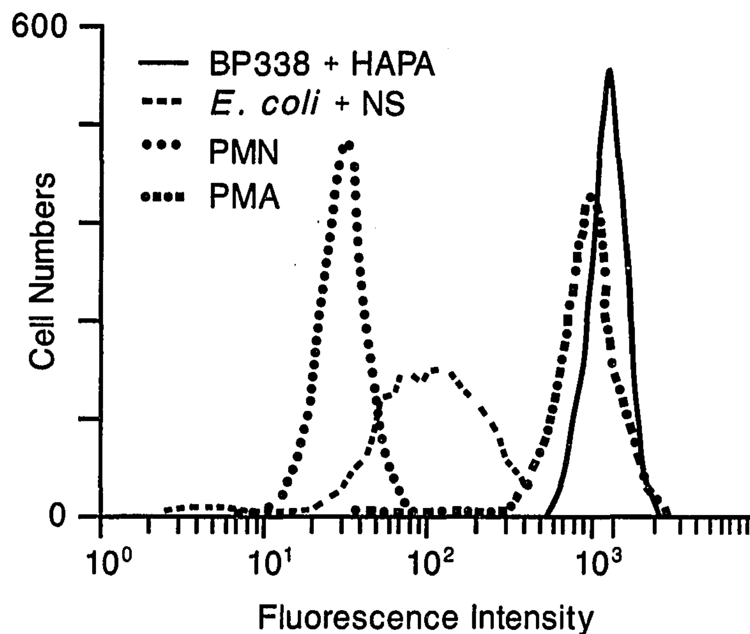

Fig. 3-10. Flow cytometric analysis of PMNL respiratory burst activity induced by *E. coli* opsonized in NS, BP338 opsonized in HAPA, and PMA. Opsonized bacteria or PMA (100 ng/ml) were incubated with PMNL loaded with DCFH-DA in a 60:1 ratio. Ten thousand events per sample were collected. The histogram of the maximum fluorescence intensity of each stimulus was graphed in comparison with the background fluorescence of the unstimulated PMNL. Histograms are from a representative experiment.

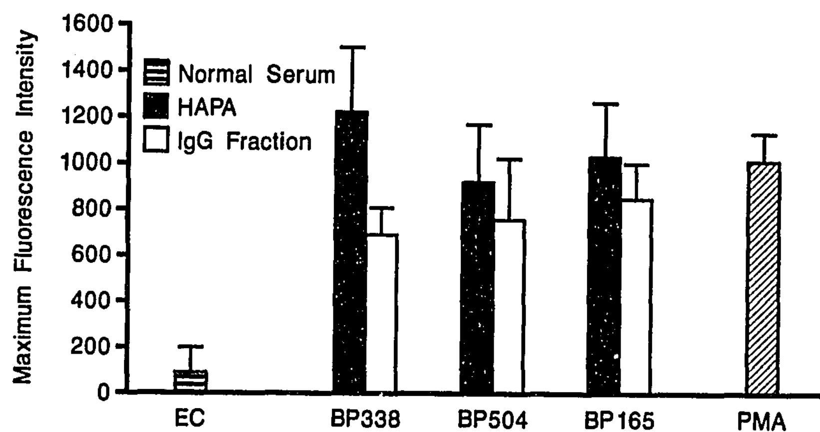

Fig. 3-11. Flow cytometric analysis of PMNL respiratory burst activity induced by *E. coli* opsonized in NS, BP338 opsonized in HAPA or IgG, and PMA. Opsonized bacteria or PMA (100 ng/ml) were incubated with PMNL loaded with DCFH-DA in a 60:1 ratio. Ten thousand events per sample were collected and the mean maximum fluorescence intensity of the stimuli were calculated. The values represent the mean ± SD of at least 3 experiments.

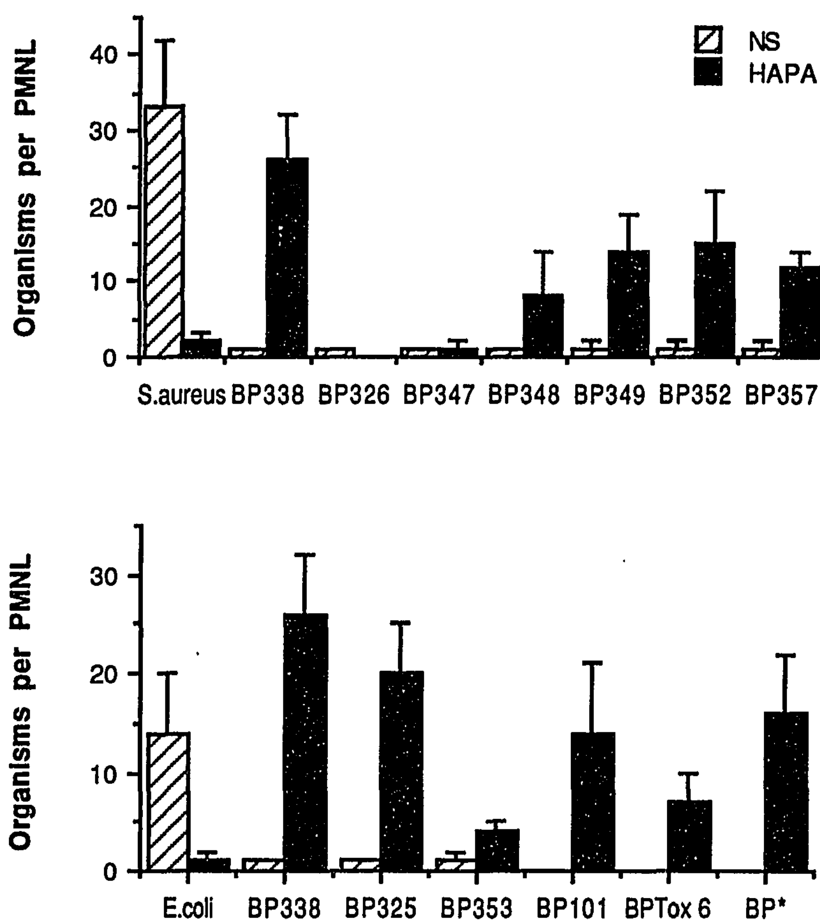

Fig.3-12. Comparison of PMNL phagocytosis of *S. aureus, E. coli,* and *B. pertussis* strains (BP* is BP101/Tox6) opsonized with NS or HAPA. Giemsa stained smears were made as described previously. Phagocytosis was determined by directly counting 50 PMNL by light microscopy and calculating the ratio of PMNL-associated bacteria per PMNL. Values represent the mean ± SD of at least 3 duplicate experiments.

TABLE 3-1. Survival of *B. pertussis* (BP) and *E. coli* after PMNL Phagocytosis

Organism	60 min	120 min
E. coli	1.2 ± 1.7	1.3 ± 1.6
BP165	79.4 ± 16.9	59.0 ± 7.6[c]
BP504	97.7 ± 34.1	76.8 ± 24.9[b]
BP338	108.6 ± 34.1	84.7 ± 17.1[c]

[a] Values presented are the percentages of surviving organisms at each time compared to their respective initial CFU (%) ± S.D. Each assay was performed at least 3 times in duplicate. All values at 60 and 120 min are significant compared to the control for that time period ($P < 0.001$).

[b] $P < 0.05$ compared to the same strain at 60 min.

[c] $P < 0.001$ compared to the same strain at 60 min.

TABLE 3-2. Survival of Mutant *B. pertussis* (BP) Strains after PMNL Phagocytosis

Organism	0 min CFU x 10^5[a]	60 min CFU x 10^5[a]	% Survival[b]
E. coli	1.3 ± 1.1	0.0038 ± 0.0086	0.29
BP338	6.0 ± 3.3	5.8 ± 4.1	96.2
BP348	6.0 ± 4.0	4.7 ± 2.6	77.5
BP325	8.5 ± 2.6	8.3 ± 2.8	97.1
BP101	7.1 ± 5.0	4.6 ± 3.1	65.2
BPTox 6	8.0 ± 5.0	4.6 ± 3.2	57.1
BP101/ Tox 6	6.4 ± 3.8	3.8 ± 1.6	59.5
BP537	21.2 ± 3.7	16.2 ± 3.6	76.4
BP347	16.7 ± 11.2	12.5 ± 2.4	74.8

[a] Values presented are the colony forming units (CFU) x 10^5 at each time ± S.D. Bacteria were opsonized in their optimal opsonin as determined by phagocytosis. Each assay was performed at least 5 times in duplicate.

[b] % Survival was calculated by dividing the 60 min CFU by the 0 min CFU then multiplying by 100.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The present study investigated the interactions of viable *B. pertussis* with human PMNL and the role of serum in the induction of phagocytosis and killing. NS from a variety of high and low titer donors were ineffective in opsonization of *B. pertussis* and induction of PMNL phagocytosis. Opsonization by HAPA and IgG fraction, which contained no complement, provided high levels of phagocytosis (Figs. 3-2 & 3-3). Addition of 5% complement to HAPA or IgG fraction generally increased phagocytosis slightly. These results were surprising since NS, HAPA, and IgG fraction had similar anti-*B. pertussis* titers by ELISA. In addition, the protein concentrations of HAPA and IgG fraction used in this study are less than the total IgG protein concentration found in NS. It is conceivable that NS contain a blocking factor that prevents anti-*B. pertussis* antibodies from binding *B. pertussis.* This blocking factor may be another antibody or other serum protein. If this blocking factor is an antibody, it must be either non-IgG or IgG diluted to nonfunctional levels. Why there is a highly significant difference in the extent of phagocytosis of BP338 induced by the IgG fraction and HAPA is unknown.

Flow cytometric analysis indicated that > 90% of PMNL having associated bacteria indeed internalized those bacteria at both the 60:1 and 1:20 ratios (Fig. 3-4). The EM studies also verified that PMNL were phagocytosing BP338. These results indicate that antibody, rather than

complement, is crucial for phagocytosis of *B. pertussis* by PMNL. This finding is particularly intriguing since we and others have established that complement is crucial for *in vitro* serum bactericidal activity against *B. pertussis* (Dolby, 1965; Dolby, 1965; Brighton, 1969). It is conceivable that complement may be an effective first line of defense of the respiratory tract during colonization of *B. pertussis*, leaving phagocytic cells to clear the remnants.

We have shown that with maximal opsonization of *B. pertussis* and the bacteria:PMNL ratio adjusted to enhance bacterial killing, *B. pertussis* is not readily killed even at 120 min incubation (Table 3-1). These results strongly suggest that *B. pertussis* has developed a mechanism(s) to circumvent phagocyte bactericidal activity and to survive intracellularly. Bacteria that survive intracellularly within phagocytes can do so by several different mechanisms. *Legionella pneumophila* (Horwitz, 1983), *Mycobacterium tuberculosis* (Armstrong, 1981; Hart, 1987), and *Clamydia psittaci* (Wyrick, 1978) survive within monocytes and macrophages by preventing phagosome-lysosome fusion. *Mycobacterium lepraemurium* multiplies in macrophage and PMNL phagosome-lysosomes because it is resistant to lysosomal enzymes (Hart, 1972). *Coxiella burnetii* has adapted to the low pH environment of the phagolysosome (Akporiaye, 1983). *Mycobacterium leprae* (Mor, 1983) and *Rickettsia tsutsugamushi* (Rikihisa, 1980; Rikihisa, 1982) escape from the phagosome into the cytoplasm.

To obtain evidence of which mechanism virulent *B. pertussis* uses to survive within PMNL, EM studies were performed to examine phagosome-lysosome fusion. Electron micrographs of PMNL with phagocytosed

B. pertussis revealed bacteria surrounded by small, tight phagocytic vacuoles similar to those seen previously by Ewanowich *et al.* (1989b). The absence of acid phosphatase staining suggested inhibited phagosome-lysosome fusion (Fig. 3-6). Quantitation of phagosome-lysosome fusion demonstrated dramatic inhibition of fusion by *B. pertussis* compared with *S. aureus*.

This is the first report of *B. pertussis* as an intracellular survivor in human PMNL. The possibility that *B. pertussis* may colonize PMNL is given credence by several previous reports regarding survival in professional and nonprofessional phagocytes. Some of the earliest studies dealing with phagocytic cell responses against *B. pertussis* were performed by Gray and Cheers (1967, 1969) using a mouse model. After intranasal inoculation, a steady state level of viable *B. pertussis* was maintained at < 1% of the pre-immune peak for almost seven weeks as determined by lung colony plate counts. *B. pertussis* was shown to persist intracellularly in alveolar macrophages. In mice challenged intracerebrally with *B. pertussis*, Hopewell *et al.* (1972) saw bacteria within the cytoplasm of ependymal cells, ciliated cells which have some phagocytic activity. The recent works of Ewanowich *et al.* (1989a, 1989b) have shown that *B. pertussis* and *B. parapertussis* invade HeLa 229 cell monolayers via microfilament-mediated endocytosis and persist, although without multiplying. In addition, *L. pneumophila* has been shown to colonize PMNL, without multiplying but without being killed (Horwitz, 1981), and *R. tsutsugamushi* has been shown to survive within PMNL (Rikihisa, 1980; Rikihisa, 1982).

It appears that inhibition of phagosome-lysosome fusion is the sole means of *B. pertussis* intracellular survival in PMNL since respiratory burst

activity is not impaired. However, the amount of PMNL respiratory burst activity induced by *B. pertussis* appears to vary depending on the assay used. Three different assays were used to measure independently both superoxide and hydrogen peroxide in order to confirm the presence or absence of respiratory burst activity. Superoxide production, as determined by reduction of ferricytochrome C, was suppressed by *B. pertussis* (Fig. 3-9). In contrast, CL assays and flow cytometry showed that whole cell *B. pertussis* induced significant levels of PMNL respiratory burst activity (Fig. 3-8 and 3-10). The flow cytometry technique measures the amount of hydrogen peroxide present within individual PMNL (Bass, 1983). CL assays monitor hydrogen peroxide released into the extracellular medium as well as that within PMNL because luminol can diffuse into phagocytic cells (Briheim, 1984). However, CL assays also monitor products from the interaction of hydrogen peroxide and myeloperoxide (Briheim, 1984). Since *B. pertussis* inhibits phagosome-lysosome fusion, CL response induced by *B. pertussis* is probably an accurate reflection of hydrogen peroxide production.

The fact that the two techniques measuring hydrogen peroxide show similar results suggests that *B. pertussis* does indeed induce PMNL respiratory burst activity. According to Pick (1986), ferricytochrome C reduction occurs by extracellular or surface superoxide. There also seems to be some disagreement as to whether or not hydrogen peroxide and other toxic oxygen products can also reduce ferricytochrome C (Arthur, 1987). Based on the data presented in this dissertation, hydrogen peroxide does not seem to be measured by ferricytochrome C reduction. Still, both virulent and avirulent *B. pertussis* strains produce superoxide dismutase and catalase

(R. Friedman, personal communication). It is possible that the superoxide produced by PMNL in response to *B. pertussis* is being rapidly degraded to hydrogen peroxide before it can leave the cell and reduce ferricytochrome C. However, this does not seem to be a problem in assessing superoxide production induced by other superoxide dismutase-producing microbes like *M. tuberculosis* (May, 1987; Zhang, 1991) and *C. burnetii* (Akporiaye, 1990).

It is surprising that intact *B. pertussis* does not inhibit respiratory burst activity since purified BPAC does inhibit this response. Confer and Eaton (1982) demonstrated that BPAC suppressed alveolar macrophage superoxide production as measured by ferricytochrome C reduction. Friedman *et al.* (1987) and Galgiani *et al.* (1988) used both ferricytochrome C reduction and CL assays to show that BPAC inhibited human PMNL respiratory burst activity stimulated by *S. aureus* or *Coccidioides immitis* arthroconidia. However, it is conceivable that the concentrations of BPAC used in those studies were greatly in excess of that to which PMNL would be subjected *in vivo.* In support of the purified BPAC studies, Mouallem *et al.* (1990) demonstrated that both purified BPAC and intact virulent *B. pertussis* increased cAMP in Chinese hamster ovary (CHO) cells. Unfortunately, CHO cells do not have a respiratory burst. It would be interesting to determine if PMNL cAMP levels and respiratory burst activity induced by intact *B. pertussis* correlate directly or indirectly.

As the apparent contradiction above demonstrates, the use of purified virulence factors can provide limited information on *B. pertussis* interactions with phagocytic cells partly because the precise concentration of any factor to which the cells would be exposed *in vivo* is unknown. In an effort to obtain

more pertinent evidence of the roles specific virulence factors play in *B. pertussis* interactions with PMNL, studies were done using spontaneous, transposon- and deletion-generated mutants (Weiss, 1983; Relman, 1989; Relman, 1990). However, the major limitation of studies using mutants appears to be imprecision of the mutation. For example, BP101, BP353, and BP325 are all considered to be phenotypically FHA$^-$. Even though BP101 has a 2.4-kilobase deletion in the structural gene, it still produces a truncated FHA that lacks one of the RGD sequences (Relman, 1989; Kimura, 1990). BP353 has transposon Tn5 inserted into either a structural or regulatory gene, but still produces minute amounts of full-sized FHA (Weiss, 1983; Brown, 1987). Spontaneous mutant BP325 produces < 1% of the FHA produced by the wild type; culture supernatants are negative for FHA by ELISA (Zhang, 1985). The same limitation is apparent with respect to mutants phenotypically PT$^-$, BP357 and BPTox6. Tn5 mutant BP357 produces small amounts of membrane-bound PT (Weiss, 1983). BPTox 6 has a 3.4-kilobase deletion of the *ptx* operon (Relman, 1989).

While the use of imprecise mutants demands careful consideration of the data, they can provide useful information. Kimura *et al.* (1990) used BP101 (FHA$^-$ deletion mutant) and its wild type parent to study colonization of the respiratory tracts of adult mice. The wild type strain colonized the lungs and the trachea over a 10-day period, as determined by dilution plate counts of the respective organs. BP101 colonized the lungs as efficiently as the wild type. In contrast to the wild type, BP101 was present in the tracheas in very low numbers until day 5 or day 10, suggesting recolonization of the tracheas. Immunoblotting of the bacteria recovered from the recolonized tracheas

demonstrated the retention of the BP101 phenotype. The authors concluded that FHA was crucial for initial tracheal colonization, but not for later tracheal colonization. A similar study had been done earlier by Weiss *et al.* (1984b) using BP353 (FHA^- Tn5 mutant). Although the same conclusions were reached by both studies, there could have been a difference in *in vivo* responses to the two slightly different mutants. For this reason, we elected to use a combination of spontaneous, transposon, and deletion mutants to further clarify the interactions of *B. pertussis* and human PMNL.

Studies of mutant phagocytosis by PMNL expanded on the importance of antibody in this process by strongly suggesting that FHA, PT, and BPAC are important ligands for opsonization by HAPA. In addition, PMNL phagocytosis does not occur in the absence of antibody bound to surface antigens because HAPA could not induce phagocytosis of several avirulent mutants (Fig. 3-12). Studies are in progress to determine the titers against these specific virulence factors present in HAPA.

Antibody made in a rabbit against an avirulent mutant was able to induce satisfactory levels of phagocytosis of all avirulent mutants tested. Since avirulent *B. pertussis* still produces LPS and tracheal cytotoxin, it is possible that the rabbit antiserum contains antibodies against these factors. It is also possible that the rabbit antiserum contains antibodies against other factors on the surface of avirulent *B. pertussis* that have not yet been characterized. These other factors may be outer membrane proteins, lipoproteins, or glycoproteins. However, regardless of the source of the antibody, once any *B. pertussis* mutant is internalized by a PMNL, it is refractory to PMNL bactericidal activity. This resistance appears to be an

inherent ability of *B. pertussis*, since even avirulent strains survive once they are phagocytosed (Table 3-2).

We were unable to determine whether BPAC, PT or any other factors may be important in survival or in the inhibition of phagosome-lysosome fusion based on the results of PMNL phagocytosis (Fig. 3-12) and killing assays (Table 3-2). Overall, however, mutations in the PT, FHA, or BPAC genes did decrease the intracellular survival of *B. pertussis* in PMNL. Yet avirulent mutants demonstrated comparable decreased survival. These results suggest that *bvg*-regulated gene products play some role in *B. pertussis* survival, but that other non-*bvg*-regulated gene products also appear to be important. What products these may be are not presently known. There is existing redundancy in *B. pertussis* virulence factors in that both PT and BPAC lead to increased cAMP levels. It would not be surprising that non-*bvg*-regulated gene products might act redundantly or synergistically with PT, BPAC, tracheal cytotoxin or other *bvg*-regulated genes to affect intracellular survival.

By what mechanism virulent *B. pertussis* inhibits phagosome-lysosome fusion is not known. BPAC may be involved in this process by causing increased intracellular cAMP levels which have been shown to occur in studies using BPAC and human phagocytes (Confer, 1982; Friedman, 1987; Galgiani, 1988). PT may also be involved, since it causes a similar increase in intracellular cAMP (Ui, 1984). However, PT requires over 12 h to cause the same cAMP increase BPAC can cause in less than 1 h (Confer, 1982). Elevated levels of intracellular cAMP have been reported to impede normal phagosome-lysosome fusion in both macrophages and PMNL (Weissman,

1975; Lowrie, 1979). In addition, intracellular calcium concentrations play an integral role in phagosome-lysosome fusion (Jaconi, 1990). Intracellular calcium stores are indirectly regulated by a PT-sensitive G protein-activated phospholipase C (Sadler, 1988).

Additional studies should begin with EM of phagocytosed mutants to determine if inhibition of phagosome-lysosome fusion is an inherent ability of all *B. pertussis* strains as is resistance to PMNL bactericidal activity. Research must continue to determine the potential role of BPAC as well as other virulence factors of *B. pertussis* in this inhibitory process. Long-term survival studies are difficult to perform in this system since the generation time of *B. pertussis* is approximately 5 h and PMNL are short-lived cells incapable of long term maintenance in tissue culture. Additional studies are in progress in Dr. Richard Friedman's laboratory to determine if intracellular survival of *B. pertussis* occurs in human monocytes and macrophages and if a similar mechanism of survival is used. Studies thus far indicate that antibody is not required for internalization of virulent *B. pertussis* and that once internalized, the bacteria do multiply.

Studies in infant mice infected via either the aerosol or intranasal route with *B. pertussis* strongly suggest that *B. pertussis* initially colonizes the nasopharynx and trachea, as seen in human pertussis. Then colonization of the lungs occurs, resulting in typically lethal pneumonia. Under many conditions, a persistent infection in the lungs developed (Goodwin, 1990; Kimura, 1990). In human pertussis the lungs are not believed to become involved nor is a persistent infection believed to occur. Yet older literature indicates that *B. pertussis* can be isolated from the lungs of fatal pertussis

cases (Olson, 1975; Weiss, 1986). If *B. pertussis* does enter the lungs during this disease, then alveolar macrophages would become a crucial defense mechanism. Since macrophages bear CR3, phagocytosis of *B. pertussis* strains bearing FHA or pertactin would not require opsonic antibody. If the macrophages were unable to kill these bacteria, they would be a source of persistent infection. Intratracheal administration of *B. pertussis* into rabbits showed that this interaction between macrophages and *B. pertussis* does occur (Saukkonen, 1991). Other intracellular pathogens have been shown to enter macrophages via integrin binding. *Histoplasma capsulatum* binds to macrophage CD18 (ß2) (Bullock, 1987) while *L. pneumophila* (Payne, 1987), *Leishmania major* (Mosser, 1987), and *Leishmania mexicana* bind to CR3 (Russell, 1988). It has been shown that CR3-mediated phagocytosis is regulated in human phagocytes. Activated macrophages and cultured monocytes will phagocytose C3b- or C3bi-opsonized particles, but phagocytosis does not induce respiratory burst activity (Wright, 1983). However, human PMNL do not phagocytose C3b- or C3bi-opsonized particles, so this mechanism does not play a role in PMNL-*B. pertussis* interactions.

If alveolar macrophage are the primary host cell for *B. pertussis* proliferation, what role do PMNL play in pertussis? When introduced by droplet infection into the upper respiratory tract, *B. pertussis* adheres to ciliated epithelial cells via FHA, surface-bound PT, fimbriae, and possibly pertactin. They begin to multiply and to produce toxins and other virulence factors. The immune response in the upper respiratory tract depends primarily on mucosal humoral response, mucociliary clearance, and sneezing,

coughing, and bronchospasms. Once *B. pertussis* multiplication and/or tracheal cytotoxin has destroyed the ciliated epithelial cells, mucociliary clearance can no longer function. Phagocytic cells and lymphocytes are present at or near the air-tissue interface throughout the upper and lower respiratory tracts. PMNL, usually located in a marginating pool in the pulmonary vasculature, are attracted into the respiratory tract by chemotactic substances. However, PT and BPAC can inhibit PMNL chemotaxis, probably via increased cAMP levels. Although BPAC is not known to have effects outside the respiratory tract, PT is well known to be circulated throughout the body. PT probably suppresses PMNL functions while they are still in the marginal pool. Although PMNL have been shown in mouse studies to enter the respiratory tract (Gray, 1967), perhaps they do so more slowly and/or in fewer numbers.

Normally, PMNL phagocytosis (e.g, internalization) is induced via Fc receptors by IgG bound to a particle. Although binding to PMNL CR3 does not trigger internalization, there is evidence that CR3 binding can synergistically increase Fc receptor binding (Scribner, 1976; Ehlenberger, 1977). IgG is found throughout the upper respiratory tract at levels approximately 1/3 the concentration of secretory IgA (Reynolds, 1988). The data in this dissertation confirms that PMNL phagocytosis of *B. pertussis* requires IgG. However, unless the host has been vaccinated, specific antibodies do not appear until days or weeks after the initiation of the infection. These facts strongly suggest that PMNL do not play a significant role in host-*B. pertussis* interactions until after the development of specific, or at least cross-reactive, antibodies. The longevity of pertussis might be explained in part by the time necessary for the

development of opsonic antibody required to induce phagocytosis by PMNL. *B. pertussis* may have many surface antigens, like FHA, PT, and BPAC, against which antibody might be produced in order to augment the likelihood of PMNL phagocytosis. This may explain why phagocytosis assays of mutant *B. pertussis* strains were unable to establish the superiority of one virulence factor over another in induction of phagocytosis.

Once opsonic antibody becomes available, PMNL take an active role in host-*B. pertussis* interactions. The act of internalization of a receptor-bound particle into a phagocytic vacuole signals respiratory burst activity to begin. This signal may be or include an increase in intracellular calcium levels (Jaconi, 1990). Internalization of Fc receptor-bound *B. pertussis* does occur. NADPH oxidase, a transmembrane complex composed of at least four distinct components, is the enzyme that reduces molecular oxygen (O_2) to superoxide (O_2^-). Activation of NADPH oxidase appears to be linked with G protein-activated phospholipase C and inositol phosphate second messenger systems (Clark, 1990). This link has not been fully characterized and so could be either a direct link such that phospholipase C activates NADPH oxidase or separate, but simultaneous, activations. However, several PMNL cytosolic proteins are phosphorylated differently by patients with chronic granulomatous disease, a syndrome characterized by defective NADPH oxidase constituents, compared with normal individuals (Hayakawa, 1986). This suggests that phospholipase C either directly or indirectly activates NADPH oxidase.

Once NADPH oxidase reduces oxygen, superoxide spontaneously dismutates to hydrogen peroxide. Superoxide alone is not particularly toxic to bacteria since all aerobic or facultative anaerobic prokaryotic and eukaryotic

cells contain superoxide dismutase. For example, the resistance of *S. aureus* to intracellular killing cannot be correlated with superoxide dismutase levels, but instead with catalase levels (Mandell, 1975). In contrast, a *Shigella flexneri* mutant with a deletion in the superoxide dismutase gene is extremely sensitive to oxidative killing by PMNL compared with its wild type parent (Franzon, 1990). Still, the major role of superoxide is considered to be as a precursor for hydrogen peroxide. The concentration of hydrogen peroxide in phagosomes can reach millimolar levels, which can be toxic to bacteria (Root, 1975). However, the major role of hydrogen peroxide is as a substrate for myeloperoxidase, an enzyme found in azurophilic granules. Once phagosome-lysosome fusion has occured, myeloperoxidase uses hydrogen peroxide to oxidize halide ions to hypohalous acids, which are highly reactive and toxic. Generally, chlorine ions are most likely to be oxidized than other halides because of their higher concentrations within the cell. Hydrochlorous acid chlorinates proteins (including those in bacterial cell walls) resulting in the formation of highly toxic chloramines. Azurophilic granules also contain a variety of proteases, hydrolases, defensins, and other antibacterial compounds which do not depend on oxygen for activity (Boxer, 1988).

B. pertussis induces normal levels of respiratory burst activity, but inhibits phagosome-lysosome fusion. If there is a link between receptor-mediated binding, phospholipase C, and NADPH oxidase then respiratory burst activity should be inhibited as was seen with purified BPAC. Yet other bacteria have been demonstrated to inhibit phagosome-lysosome fusion or respiratory burst or both or neither. For example, *M. tuberculosis* prevents

phagosome-lysosome fusion, but has no effect on respiratory burst activity (May, 1987). In contrast, *Brucella abortus* inhibits respiratory burst activity, but not phagosome-lysosome fusion (Riley, 1984). *L. pneumophila* inhibits both respiratory burst activity and phagosome-lysosome fusion (Friedman, 1982; Horwitz, 1983). This suggests that NADPH oxidase activation occurs simultaneously, but separately, from phospholipase C activation of inositol phosphates and release of intracellular calcium stores. In addition, the fact that phagosome-lysosome fusion does not occur in response to internalized *B. pertussis* explains how they can survive intracellularly. Prior to fusion with lysosomes, the phagosome contains only superoxide and hydrogen peroxide. *B. pertussis* produces both SOD and catalase to breakdown these oxidative products into oxygen and water, allowing its survival in the phagosome.

The residency of *B. pertussis* within PMNL may not be simply a mechanism for avoidance of complement killing. It also does not appear likely that *B. pertussis* multiplies within PMNL because of the long generation time of the bacteria and the short life-span of PMNL. PMNL can also modulate the immune response, although to a much less degree than macrophages. PMNL lysosomes contain complement activators, plasminogen activator, and lactoferrin. Lactoferrin is thought to be involved with controlling toxic oxidative products released during inflammation. There is also evidence that lactoferrin can activate macrophages (Lima, 1985). Via a PT-sensitive G protein-mediated phospholipase A_2, arachidonic acid is converted through the lipooxygenase pathway to leukotrienes and several HETE compounds. LTB_4 stimulates chemotaxis and lysosomal enzyme release of PMNL (Hensler, 1991). By the same or a similar mechanism used to prevent phagosome-

lysosome fusion, *B. pertussis* may prevent exocytosis of lysosomal contents thus suppressing the immune response. PT has also been shown to reduce the number of N-formylated peptides receptors on PMNL (Hensler, 1991).

Intracellular survival of *B. pertussis* within PMNL and alveolar macrophages might explain many unanswered questions regarding pertussis. Pertussis is a strictly human disease, yet there are no known carriers and no known reservoirs or vectors of the causative bacteria (Olson, 1975; Manclark, 1984). If *B. pertussis* attains an intracellular habitat, a carrier/reservoir state may be the source for new infections. Dead and dying PMNL in the respiratory tract are generally believed to be removed by mucociliary clearance. Perhaps parasitized PMNL are expelled during paroxysmal coughing, providing a convenient package of inoculum to susceptible individuals. Indeed, there is increasing evidence that adults frequently demonstrate asymptomatic or atypical pertussis and that adults are often the source of infection for children who develop pertussis (Robertson, 1987; Thomas, 1987; Long, 1990). In addition, there are several reports of pertussis in individuals, particularly adults, infected with the human immunodeficiency virus (Ng, 1989; Doebbeling, 1990).

As with other microbes that parasitize PMNL and macrophages, cell-mediated immunity is likely to be of greater importance in clearance of *B. pertussis* than humoral immunity. There is evidence in the literature that suggests that antibody titers, particularly against PT, do not always indicate the degree of protection of an individual from pertussis. The stimulation of only humoral immunity may be one of the reasons some experts feel that vaccination does not provide protection as long-lasting as does immunity from

natural infection (Lambert, 1965; Nelson, 1978). Recent studies of adults who had had pertussis as children demonstrated T cell clones that were reactive against FHA, pertactin, or PT (De Magistris, 1988). Another study demonstrates that the transfer of T cells from mice that had recovered from aerosol pertussis infection to immunosuppressed mice were able to rapidly clear a subsequent challenge. Immune T cells proliferated in response to FHA, pertactin, and agglutinogens, but did not clearly respond to active PT and did not respond to PT toxoid (Mills, 1990). Assessment of T cell response during pertussis, after pertussis, and in response to vaccination is the newest area of pertussis research.

An intracellular habitat for *B. pertussis* might also explain why the isolation of these bacteria from the nasopharynx during the paroxysmal stage is so difficult (Olson, 1975; Manclark, 1984). Convalescence from uncomplicated pertussis is slow, gradual, and of long duration. Spontaneous paroxysmal coughing may last up to 6 months and isolated coughing bouts may recur with any respiratory infections for up to two years after acute pertussis (Olson, 1975; Manclark, 1984). It is conceivable that instead of being cleared early in the infection the bacteria remain intracellular, protected from serum bactericidal activity. From this protected intracellular site, toxic products and bacteria may be released over an extended period of time causing a more lengthy disease process.

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