THE ROLE OF CAPSULAR MATERIAL OF *PASTEURELLA HAEMOLYTICA*, TYPE 1, IN BOVINE PNEUMONIC PASTEURELLOSIS

By

MARTHA JOY GENTRY

Bachelor of Science
Kansas State University
Manhattan, Kansas
1969

Master of Arts
Indiana University
Bloomington, Indiana
1971

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1984

THE ROLE OF CAPSULAR MATERIAL OF PASTEURELLA HAEMOLYTICA, TYPE 1, IN BOVINE PNEUMONIC PASTEURELLOSIS

Thesis Approved:

Thesis Adviser

Major Adviser

Dean of the Graduate College

ACKNOWLEDGMENTS

There are many people without whom this thesis would not have been written, and to whom I am very grateful. I would like to thank Dr. Richard E. Corstvet for initially encouraging me to enroll in the doctoral program and stimulating my interest in bovine respiratory disease. During the course of these studies I feel that I have grown and changed a great deal. My thanks to Dr. Helen S. Vishniac, who forced me to see that I needed to; to Dr. Roger J. Panciera, who told me that I had to; to Dr. John T. Homer, who always made me feel that I could; and to Dr. Sidney A. Ewing, who helped me make it happen. My special thanks to Dr. Anthony W. Confer, who somehow seemed to know when I needed a pat on the back or a kick from behind, and was not afraid to apply either.

I am grateful to several other people whose support ranged from media-making to hand-holding. They are: Lisa Corstvet, John Rummage, Paul Newman, Carolyn Gresham, Derek Mosier, René Simons, Judy Kreps, Chad Faulkner, Rhonda Smith, Carol Emerson, and Janet Durham.

Finally, I would like to thank my family, who shared both the troubles and the triumphs with me. My husband, Jim, had to set aside his own work at times to put mine first, and my daughters, Robin and Erin, had to learn to do many things for themselves.

This research was supported in part by the Oklahoma Agricultural Experiment Station Research Projects #1438 and #1725, as well as by a United States Department of Agriculture Special Grant #83-CRSR-2-2195.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

REVIEW OF THE LITERATURE AND STATEMENT OF THE PROBLEM

The Disease

Shipping fever, or pneumonic pasteurellosis of cattle has been reported to be the most prevalent and serious disease of feedlot cattle in North America (Thomson, 1980). In a survey of 10 veterinary practices, each involving 35,000 to 225,000 cattle annually, Jensen (1968) found that 40% to 80% of diseases encountered involved the respiratory system. In 1966 it was estimated that over 100,000 cases of bovine respiratory disease occurred in the United States and resulted in losses of approximately $75,000,000 (Abinanti, 1968). A more recent estimate of financial loss due to the disease was one third of a billion dollars annually (Morter et al., 1982). Actual mortality rates from the disease are generally low, particularly if adequate treatment is administered when clinical signs first appear. Thus the greatest costs of the disease involve loss of weight and condition and expenses connected with preventive programs and treatment measures, rather than actual death losses.

The term shipping fever was first used to describe a large variety of respiratory ailments among cavalry horses (Turner, 1916). The same term was also used to describe respiratory diseases of cattle in the

early 1900's (Hardenbergh and Boerner, 1918). There was a great deal of confusion at that time about the etiology of the disease as well as about the distinction between various syndromes associated with the name. Literature references to diseases known variously as transport fever, fibrinous pneumonia, stockyard pneumonia, and exposure disease, all appear to refer to the nebulous array of diseases that eventually became known as the bovine respiratory disease complex. It is now known that this complex of diseases consists of at least three different clinical entities: (1) Enzootic pneumonia of calves, (2) shipping fever, and (3) atypical interstitial pneumonia (Lillie, 1974). Only within recent years has the term pneumonic pasteurellosis come into common use to indicate the acute, fulminating lobar fibrinous pneumonia associated with Pasteurella haemolytica infection in calves (Jubb and Kennedy, 1970).

P. haemolytica and P. multocida have been known to be associated with bovine respiratory disease for many years. However, their significance as pathogens was not fully appreciated because of the complex etiology of the disease. As early at 1895, Moore (1895) reported that Pasteurella spp. were present as commensals on the respiratory mucous membranes of normal animals (cattle, sheep, swine, dogs, and cats). Jorgenson (1925) later studied 250 cattle and confirmed these findings. In addition, he isolated the organism at necropsy from 4 animals that died of causes other than pneumonia and without lung lesions. He could not distinguish the bacteria isolated from healthy cattle from those isolated from diseased animals. Scott and Farley (1932) performed necropsies on 26 cattle from different herds affected with shipping fever. An organism called Pasteurella boviseptica at that time (see section on "The Organism") was isolated from 81% of the cases. It was also the only organism isolated in pure culture in cases diagnosed

as shipping fever. In contrast, none of 83 normal lungs from a packing house yielded P. boviseptica upon culturing. They thus concluded that P. boviseptica was the most important bacteriological factor in shipping fever. However when taking Jorgenson's work into account they also stated, "while P. boviseptica may be the primary bacteriological factor of shipping fever, the causative factor in the production of the disease should be looked for elsewhere" (Scott and Farley, 1932, p. 177).

A major barrier to elucidating the role of Pasteurella organisms as contributors to the disease process as well as the efficacy of protective measures has been the difficulty in artificially reproducing the disease. Jorgenson (1925) was able to infect two cows experimentally after they had been transported and chilled. One was infected by inhalation of an aerosolized culture and the other by feeding of 250cc of a 24 hr broth culture of "P. boviseptica". Carter (1956b) exposed 19 calves, most intranasally, with agar or broth cultures or lung tissues infected with P. haemolytica. The calves were not purposely stressed. Eight of the 19 animals showed mild to moderate clinical signs of pneumonia. P. haemolytica was recovered at necropsy from the lungs of all of these animals. He postulated that preexisting inapparent chronic pneumonias, indicative of viral infection, were present in some of his experimental calves and contributed to the severity of the P. haemolytica infections.

During the 1950's, attention began to be focused on the combined effects of P. haemolytica and other infectious agents, and environmental stresses. Palotay and Christensen (1959) reported fever, respiratory distress, and one death in calves as a result of concurrent intratracheal inoculation of P. haemolytica and the sporadic bovine

encephalomyelitis agent. The next year Collier et al. (1960) superimposed a P. haemolytica infection by aerosolization of cattle infected 3 days previously with infectious bovine rhinotracheitis (IBR) virus. In that way, they were able to produce clinical pneumonia in 2 of 4 animals and a severe febrile reaction in all four. Jericho et al. (1976) induced disease in 11 of 20 calves with a combination of IBR virus, P. haemolytica, and stress (including weaning the day of virus exposure and daily temperature fluctuations of 77° F to 8° F). More consistent disease production has been achieved recently by such methods as intrabronchial injection of 10^6 - 10^7 live P. haemolytica organisms/ml (Wilkie and Markham, 1979), and transthoracic intrapulmonic injection of $5x10^9$ live organisms/lung (Panciera and Corstvet, 1984).

Current theories on the initiation of pneumonic pasteurellosis agree that P. haemolytica is the most important agent in the final stages of the disease. Stresses of one kind or another (transportation, weaning, overcrowding, social acclimation, fatigue, temperature and humidity extremes, food and water deprivation, castration, dehorning, worming, vaccination procedures, etc.) are usually given credit for predisposing to most outbreaks. One development during stress is an increased production of adrenal corticosteroids which have been shown in vitro to impair ingestion by phagocytes (Stossel, 1973), and to block the release of chemotactic factors from alveolar macrophages and the binding of such factors to PMN's (Dyer, 1982). A number of infectious agents other than pasteurellae also have been implicated as predisposing factors. These include bovine herpes virus type I (IBR) (Jericho and Langford, 1978; Filion et al., 1983)., parainfluenza-3 virus (Baldwin et al., 1967), bovine respiratory syncytial virus (Rossi

and Kiesel, 1974), bovine virus diarrhea virus (Roth et al., 1981), Mycoplasma spp. (Hamdy et al., 1958; Hamdy, 1968), and others.

A convincing hypothesis on the role of viral agents was put forth by Collier (1968). He reasoned that:

> . . . the pasteurellae have difficulty in establishing infection on intact epithelial membranes but enjoy an increasingly favorable growth medium once host epithelial cellular debris and serous fluid have been elicited by virus (p. 826).

Thus he felt that viral infection should simply be considered as an example of "physiological stress." Other investigators have established more active roles for viruses in depression of host phagocytic defense mechanisms. Attachment of influenza virus to rodent polymorphonuclear leukocytes (PMN) has been shown to inhibit leukocyte glycolysis and depress subsequent ingestion and killing of bacteria (Sawyer, 1969). Parainfluenza I virus infection of murine alveolar macrophages was also shown to retard ingestion of yeast cells and prevent phagosome-lysosome fusion needed for efficient intracellular killing of these fungi (Jakab et al., 1980). A very different effect of virus on alveolar macrophages was suggested by Markham et al. (1982), who stated that,

> . . . the effect of virus on macrophages may not be due to a direct effect on the phagocytic or killing capability of the cell itself, but rather on its ability to recruit additional cells, like the PMN, to aid in clearance of the bacterial inoculum (p. 288).

Such reports suggest possible mechanisms of virus-induced suppression of phagocyte function which could contribute to postviral bacterial infections.

Whatever the predisposing factors, the critical event in production of pneumonic pasteurellosis appears to be rapid growth of P. haemolytica, or more rarely P. multocida, in the nasopharynx and eventually in the lungs, due to gravitational drainage or inhalation of droplets within inspired air (Thomson, 1981). In a comparison of nasal swabs taken from 200 apparently healthy cattle and 59 cattle which had been diagnosed as having clinical shipping fever, Pasteurella spp. were found in only 3% of the normal calves, but in 60% of those with shipping fever (Hoerlein et al., 1961). During and after shipment and congregation of cattle in feedlots, both the number of animals carrying P. haemolytica and the numbers of organisms per animal increases, as determined by cultures of nasal swabs (Thomson, 1980). The organism is thought to be spread from animal to animal by droplet nuclei produced during coughing and labored respiration of sick animals, or by contamination of feed and water containers with nasal exudates (Lillie, 1974). As the number of organisms on the nasal mucosa increases, the number of organisms presented to the lung also increases, so that eventually resistance mechanisms are overwhelmed. Biberstein and Thompson (1965) concluded that the virulence of P. haemolytica depended on its ability to grow rapidly in an animal from a small inoculum to a population that is toxic.

Originally it was thought that the toxicity of P. haemolytica was due to release of endotoxin from lysed organisms (Carter, 1968; Lillie, 1974). Keiss et al. (1964) extracted endotoxin from a bovine strain of P. haemolytica and concluded that endotoxin comprises approximately 12.25% of the dry weight of the organism. Those investigators concluded that when such large numbers of organisms are present, as in the lungs of cattle with shipping fever, endotoxin effects, including death, may

be expected. It was more strongly stated by Lillie (1974, p. 237) that "the clinical signs of 'shipping fever', excluding the obvious hypoxia and dyspnea resulting from loss of a large percentage of functional lung parenchyma, may be explained by endotoxicity." As early as 1960, G. R. Smith (1960) reported that a heat-killed (55° C for 30 min) broth of P. haemolytica could kill lambs at only a slightly higher multiple dose than that of a living culture. The signs produced were thought to be due to endotoxin.

Within the last few years, several investigators have shown that P. haemolytica toxicity is primarily due, rather, to a product of live, metabolically active organisms. The toxicity has also been shown to be species specific, in that it is limited to ruminants (Kaehler et al., 1980b) and cell specific, in that it shows greater toxicity for leukocytes and macrophages than for spleen cells or erythrocytes (Shewen and Wilkie, 1982). Live, but not heat-killed, bacteria were found to be toxic to cultured bovine alveolar macrophages (Benson et al., 1978). Similarly, live but neither heat-killed nor X-irradiated organisms were shown to kill bovine blood monocytes (Kaehler et al., 1980a). Markham and Wilkie (1980) reported that formalin-killed P. haemolytica caused bovine cultured alveolar macrophages to detach from cover slips, but they were unable to show similar killing of suspended macrophages by a ^{51}Cr-release assay. Further investigations revealed that P. haemolytica cultures in the logarithmic growth phase were more toxic than stationary-phase cultures for bovine neutrophils (Berggren et al., 1981; Markham et al., 1982).

The exotoxin produced by live organisms is now known as P. haemolytica cytotoxin. It was first alluded to by Benson et al., (1978),

who reported a "toxic factor" present in bacteria-free filtrates from organisms grown in tissue culture medium M199 containing 10-20% fetal bovine serum. Logarithmic-phase cultures of P. haemolytica were later reported to produce a soluble "toxin" that was released into the medium when the organisms were grown in RPMI 1640 medium (Berggren et al., 1981). The toxin was shown to kill bovine neutrophils, causing them to lose their phagocytic capability. Because it affected phagocytes, the authors stated that it should be considered a virulence factor. The same group of investigators performed the first characterization studies on the toxin itself. They showed a correlation between cytotoxin production and culture age; production peaked during the logarithmic growth phase and then decreased during the stationary phase of growth (Baluyut et al., 1981). They reported that molecular weight of the cytotoxic substance was 300,000 or more, and that it was heat labile, oxygen stable, and susceptible to extremes of pH. They further found that it was inactivated by trypsin, and did not contain detectable endotoxin or hemolytic activity. Partial purification of P. haemolytica cytotoxin by Himmel et al. (1982) resulted in a 150,000 molecular weight protein which reacted by double immunodiffusion to antiserum prepared against all 12 serotypes of P. haemolytica, but not to antiserum prepared against 15 different serotypes of P. multocida. Those researchers felt that the protein was capsular. Another group studying enzyme activity in crude cytotoxin preparations demonstrated both a neuraminidase and a novel protease (Otulakowski et al., 1983). They suggested that the protease might have a function in unmasking receptors for cytotoxin on phagocytes, or in cleaving and thus activating the toxin itself.

Further purification and characterization of cytotoxin is obviously needed. At this time, however, even without more refined definition it appears to be an important factor in development of pneumonic pasteurellosis, because it can impair production of chemotactic factors by pulmonary alveolar macrophages and kill bovine phagocytes (Markham et al., 1982).

The Organism

In 1885, Kitt and coworkers described an organism associated with the shipping fever complex (Kitt et al., 1885), and they named it Bacterium bipolare multocidum. The organism was indistinguishable culturally from that which had been described five years earlier by Pasteur (1880) as the cause of fowl cholera, as well as from the bacterium associated with swine plague and septicemia of rabbits. Because it was believed that these organisms were the same, they were grouped together under the name Bacterium septicemiae hemorrhagicae. Trevisan (1887), however, proposed that the organisms be considered as separate species with a common generic name, Pasteurella, in honor of Pasteur. Accordingly, Lignières (1901) presented a system of classification, earlier proposed by Flügge (1886), suggesting that the strains be named according to the animal species in which they caused disease, i.e., P. boviseptica, P. aviseptica, P. suiseptica, etc.

Jones (1921) examined many organisms isolated from cattle with hemorrhagic septicemia and divided them into three groups which were distinguishable by agglutination reactions. Those which could hemolyze bovine and equine erythrocytes he called Bacillus bovisepticus Group 1 (also called P. boviseptica by other workers). The bacteria in this

group were later given the name Pasteurella haemolytica by Newsom and Cross (1932) in reference to their hemolytic capability. Thus, P. haemolytica was recognized as a separate entity from the organism which became known as Pasteurella multocida. An organism first called P. mastitidis (because it was repeatedly isolated from cases of mastitis in ewes) is now also included under the name P. haemolytica.

It is now known that P. haemolytica is involved as an etiologic agent of pneumonias in cattle, sheep, goats and swine, as well as septicemia of lambs and mastitis of ewes (Carter, 1968). Edwards (1959) reported the organism to be the cause of a septicemia in neonatal pigs and Maplesden and Carter (1955) reported it in a case of bovine mastitis. In addition, it has been implicated as a cause of central nervous system lesions in a calf (Goto and Itakura, 1975). In general, P. haemolytica is not highly pathogenic for the species in which it is known to cause disease. This is attested to by the relatively high carrier rates found in healthy animals (Carter, 1968). It is also only mildly pathogenic or nonpathogenic for the common laboratory animals, including mice, rats, guinea pigs, rabbits, cats, and dogs.

Elucidation of the different serologic types of P. haemolytica lagged far behind that for P. multocida. Because many fresh P. haemolytica isolates proved to be inagglutinable, Carter (1956a) decided to type the organisms by an indirect hemagglutination (IHA) technique which had been used successfully on P. multocida in the early 1950's. The IHA test utilized agglutination of erythrocytes to which were adsorbed specific lipopolysaccharide capsular substances from the organisms. Because all of the 61 bovine and ovine strains which Carter tested were found to be serologically homogeneous, he mistakenly assumed

that pathogenic strains were of the same serologic type. However, Smith (1959) examined the cultural, biochemical, and pathological characteristics of strains of P. haemolytica isolated from cases of ovine pneumonia and divided them into 2 groups which he called biotype A and biotype T based on their ability to utilize arabinose or trehalose. Further studies using a modified IHA test on strains isolated from healthy and diseased cattle and sheep led Biberstein et al. (1960) to divide the species into 10 capsular serotypes designated by arabic numbers. Whereas all of the different serotypes were recovered from sheep, all but 2 of the bovine isolates tested were of type 1. One of the other bovine strains was of type 2 and the remaining isolate was untypable. The number of serotypes was later expanded to 12 and all 12 types were grouped under Smith's A and T biotypes according to the following distribution (Biberstein and Gills, 1962; Biberstein and Thompson, 1966):

Biotype A contains serotypes 1, 2, 5, 6, 7, 8, 9, 11, and 12

Biotype T contains serotypes 3, 4, and 10

Although the IHA test is both sensitive and specific for serotyping P. haemolytica, it is labor intensive because antigens must be prepared from each culture to be tested and then adsorbed to erythrocytes. Therefore a rapid plate procedure for serotyping P. haemolytica was developed by Frank and Wessman (1978). Their method can be performed in a matter of minutes using colonies grown on blood agar plates with essentially the same results as IHA testing.

In addition to the typing systems mentioned, differences in cultures relating to their degree of encapsulation were noted.

Biberstein et al. (1958) observed two different types of colonies in each of three P. haemolytica cultures isolated from lambs. The colonies differed in morphology, ability to take up crystal violet dye, adherence to the agar surface, and agglutinability. They determined that the basic difference between the two colony types was the amount of "surface substance" possessed and thus designated the variants S for smooth (with abundant surface substance) and R for rough (deficient in surface substance).

Wessman (1964) studied broth cultures of the organism and found that both smooth and nonsmooth variants occurred within the population. He reported that, as the culture aged and the amount of available oxygen decreased, smooth cells died rapidly and "were replaced by nonsmooth variants" (Wessman, 1964, p. 356). He also noted a shift from smooth to nonsmooth organisms on agar slants stored at 5° C. Corstvet et al. (1982a) later reported that the majority of P. haemolytica organisms are encapsulated in the early logarithmic phase of growth but that the capsular material appeared to be sloughed off the cells as the culture aged so that most stationary-phase organisms were no longer encapsulated. Perhaps the age-dependent nature of encapsulation explains the dissociation from smooth to nonsmooth organisms observed by Wessman (1964). An association between smoothness of cultures and virulence was also noted. Wessman (1964) found that the smooth variants in his broth cultures were more pathogenic for mice than were the rough variants, even though the two were indistinguishable in biochemical characteristics. Lilli (1977) also reported that it was the smooth colonial variant of P. haemolytica, serotype 1, which was almost always associated with bovine respiratory disease.

Prevention of the Disease

Control of pneumonic pasteurellosis of cattle depends upon (1) reduction in stress factors known to predispose animals to the disease, (2) reduction of exposure to infectious agents involved in the disease, and (3) immunological enhancement of resistance to the infectious agents.

Control of stress factors may partially be achieved, even though transport of animals is a necessity. Dyer (1982) proposed several management practices designed to minimize stresses on beef cattle at the time of weaning, sale, and transport. Such "preconditioning programs" include weaning calves from dams at least one month prior to transport, early introduction to the post-transport diet, limiting transportation to the shortest possible distances, use of rest stops during which the animals have access to food and water, and planning of transportation times to avoid excessively inclement weather.

Reduction of exposure to infectious agents may depend primarily on minimizing the mixing of animals purchased from different sources for the first 45 days after arrival in a feedlot, during which time losses from respiratory disease are highest (Dyer, 1982). In addition to the social stress factor induced, mixing groups of cattle has been associated with widespread shedding and exchanging of viruses in the first 60 days post arrival at the feedlot (Irwin et al., 1979). Animals housed out-of-doors should be provided with some shelter from damp weather or excessive wind or sun. Indoor facilities should be equipped with proper air exchange systems to remove aerosols of pathogenic organisms shed by infected individuals, remove warm air containing

excessive moisture, and allow for even, draft-free intake of fresh air (Dyer, 1982).

Immunological enhancement of resistance to infectious agents has been achieved classically through the use of vaccines (containing live or killed organisms), bacterins (containing killed organisms, by definition), or aggressins (crude supernatants from bacterial cultures). The earliest bacterins used against shipping fever were prepared from P. multocida only. Carter (1957), however, suggested that shipping fever bacterins should contain both P. multocida and P. haemolytica, type 1. He also stressed that only "freshly isolated capsulated cultures" and organisms harvested after short incubation periods (6-8 hr) should be used to favor "maximum antigenicity" (Carter, 1957). In several trials where Pasteurella bacterins were used, they were reported to have a protective effect against development of respiratory disease (Palotay et al., 1963; Hamdy and Trapp, 1964; Matsuoka et al., 1972; Morter et al., 1982). In general, however, use of Pasteurella bacterins in controlled studies has not proved efficacious. It was reported in two early studies that Pasteurella aggressins provided better protection for cattle than did bacterins (Buckley and Gochenour, 1924; Miller, 1927). In another study, a dramatic increase in mortality rates of animals given either aggressin or bacterin, or both preparations in combination was reported (Farley, 1932). In later studies also, bacterin-vaccinated or bacterin plus virus-vaccinated animals were shown to have equal or greater death losses, weight losses or disease prevalence than control animals (Hamdy et al., 1965; Woods et al., 1974; Friend et al., 1977; Martin et al., 1980; Amstutz et al., 1981; Bennett, 1982). Wilkie et al. (1980) vaccinated calves either intrabronchially or

subcutaneously with formalin-killed P. haemolytica and then challenged the calves with live P. haemolytica intrabronchially. They found that after challenge, respiratory rates and macroscopic pulmonary lesions were greater in calves vaccinated subcutaneously than in control animals. Immunization by the intrabronchial route was not associated with adverse effects and was thought to provide a moderate degree of protection. Those investigators theorized that parenteral immunization with the killed organism promoted production of IgG antibodies which were opsonic and therefore enhanced phagocytosis by macrophages. The same group had previously shown (Markham and Wilkie, 1980) increased phagocytosis to be detrimental to phagocytes. They felt that intrabronchial immunization, however, stimulated a non-opsonic IgA response that was mildly protective and not deleterious to the macrophages. Another theory on failure of bacterins to provide effective protection against shipping fever is that parenteral exposure to killed organisms may fail to generate antibody capable of neutralizing P. haemolytica cytotoxin (Dyer, 1982). Review of data such as these on the use of bacterins may be summed up by Wilkie's (1981) statement that

> In view of the historically extensive use of these bacterins, the continued high prevalence of fibrinous pneumonia with isolation of P. haemolytica and the known potential of vaccination as a general procedure to very successfully protect against several important veterinary diseases, it seems safe to assume that Pasteurella bacterins do not have obvious usefulness in control of shipping fever (p. 113).

In recent years, attention has been turned toward other types of Pasteurella vaccines. Potassium thiocyanate or sodium salicylate capsular extracts from P. haemolytica have been shown to be protective against experimental pneumonia in both sheep (Gilmour et al., 1983;

Mukkur et al., 1983) and cattle (Yates et al., 1983). Other studies have shown that cytotoxin preparations from the organism are antigenic for rabbits (Shewen and Wilkie, 1983a) and cattle (Baluyut et al., 1981; Opuda-Asibo et al., 1983). In a study of sera obtained from beef cattle at necropsy, the cytotoxin-neutralizing activity was significantly lower for sera from calves dying of fibrinous pneumonia than from calves dying for reasons other than pneumonia (Shewen and Wilkie, 1983b). Gentry and Confer (1983) also showed that the cytotoxin-neutralizing capacity of serum from calves unexposed, naturally exposed, or exposed by vaccination to live P. haemolytica was significantly correlated to ability of the calves to resist an experimental P. haemolytica challenge. These results indicate that induction of the ability to neutralize P. haemolytica cytotoxin may be an important part of an effective immunization program against pneumonic pasteurellosis.

Immunization with live P. haemolytica, either parenterally or by aerosol, has been shown recently to enhance resistance to a trans-thoracic, intrapulmonic challenge with the organism (Corstvet et al., 1978; Confer et al., 1984a; Panciera et al., 1984). In a subsequent series of comparative studies, live vaccines were consistently more effective than bacterins in enhancing resistance to experimentally-induced disease (Confer et al., 1984b). The superiority of immunizing capacity of live compared to killed organisms may result from the multiplication of live bacteria in the host. Live preparations would be more likely to present the immune system with antigens, such as those found in capsular material or cytotoxin, which may be absent or altered in killed preparations. Future immunization attempts should thus utilize live organisms or antigen preparations from them.

Interactions of Gram Negative Bacteria with Mammalian Serum Factors

The capacity to kill gram negative bacteria is associated with serum from a variety of warm-blooded and cold-blooded animals, including fish (Taylor, 1983). Killing of susceptible bacteria is mediated by complement which may be activated by either the classical or alternate pathway (Taylor and Kroll, 1983). Classical complement activation is generally accomplished by the interaction of antibody and surface antigens on the organisms (Atkinson and Frank, 1980). The classical pathway may also be activated directly by the lipid A region of lipopolysaccharide (Loos et al., 1978), but lipid A is normally not exposed on the surface of gram negative bacteria. The alternate pathway is antibody independent and may be activated by a number of surface structures found on bacteria, such as cell walls and lipopolysaccharides, as well as by aggregated immunoglobulins (Atkinson and Frank, 1980). Activation of complement by either pathway eventually leads to deposition on the cytoplasmic membrane of complement factors C5b-C9. This aggregate, called the membrane attack complex, is responsible for the irreversible damage that causes death of the organism (Taylor and Kroll, 1983). Bacteriolysis, when it occurs, is carried out by lysozyme. However, lysozyme bacteriolysis can only occur after the membrane attack complex has been integrated into and has destroyed the integrity of the cell membrane (Taylor, 1983).

When antibody-mediated activation of complement occurs, extremely small amounts of antibody are required, making the bactericidal reaction much more sensitive for determining the presence of antibody than the agglutination reaction (Taylor, 1983). IgM is more efficient at

activating complement by the classical pathway than is IgG because one molecule of IgM is sufficient to activate one molecule of the C1q component of C1, whereas two molecules of IgG are necessary and they must be bound in close proximity (Taylor, 1983). Certain IgG subclasses (depending on the species of animal examined) are not capable of activating complement to a significant extent. IgA is not only thought to be inactive in the bactericidal reaction, but is known to inhibit the bactericidal effect of IgM and IgG in certain cases, presumably by binding to available antigenic sites in a competitive blocking reaction (Rowley, 1973).

Certain bacteria are able to resist the bactericidal action of serum. To date there is no evidence that such organisms are resistant because of an ability to produce extracellular products that neutralize or destroy the functional integrity of the complement components (Taylor, 1983). There is evidence that the outer cell membrane is involved in the serum resistance of some gram negative organisms. Pretreatment of serum resistant cells with Tris and EDTA, which releases much of the lipopolysaccharide complexed to the outer membrane and also lowers its permeability barrier, has been shown to increase the sensitivity of the cells to serum bactericidal action (Reynolds and Pruul, 1971). Treatment of certain serum-resistant enterobacteria to Polymyxin B, which adversely affects the integrity of the outer membrane, is also known to cause the organisms to become serum sensitive (Fierer and Finley, 1979). Taylor (1983) proposed that

> . . . serum resistance does not result from a block in the activation of the complement cascade. Rather, MAC's [membrane attack complexes] formed on the surfaces of serum-resistant strains are not effectively inserted into the

> bacterial membranes and are released without causing lethal damage (p. 63).

Smooth strains of gram negative bacteria possessing lipopolysaccharide with core units highly substituted by O antigen side chains are generally found to be more resistant to serum killing than rough strains lacking the O antigen component of lipopolysaccharide (Rowley, 1968; Reynolds and Pruul, 1971; Taylor, 1975). It has been proposed that the serum resistance of smooth strains is due to interference by the O antigen polysaccharides with the formation, attachment, or subsequent activity of the membrane attack complex (Taylor, 1983).

It has frequently been observed that bacteria in the logarithmic phase of growth are more sensitive to serum killing than are those in the lag or stationary phases (Davis and Wedgwood, 1965; Rowley, 1973; Taylor and Kroll, 1983). Rowley (1971) proposed that the increased sensitivity of rapidly dividing organisms could be due to an inability of sugar transferases, which link together the repeating oligosaccharide units of the smooth O antigens, to keep pace with cell wall production. Such cells would have exposed areas of rough polysaccharide unprotected by the smooth O antigen. Another theory is that a certain amount of cellular biosynthetic activity or energy is necessary for serum killing, and lag- or stationary-phase cells are not as metabolically capable of producing the needed components (Taylor and Kroll, 1983).

Some differences in sensitivity of gram negative bacteria to the bactericidal effects of serum have been shown to depend on environmental conditions and the metabolic state of the organisms being tested. The antigenic composition of a bacterial culture varies greatly depending on the environmental conditions imposed, including temperature

and availability of oxygen, nutrients, energy sources, etc. Phenotypic variations due to culture conditions thus can affect the serum sensitivity of an organism by influencing the biosynthesis of cell surface structures and the metabolic state of the cells (Taylor, 1983).

The importance of temperature regulation in serum bactericidal assays has been reported (Osawa and Muschel, 1964). Temperatures at or near optimum for bacterial growth should be used because suboptimal or elevated temperatures can cause reductions in killing rates (Kato and Bito, 1978). Those authors hypothesized that membrane fluidity, permitting a high degree of molecular motion, is obligatory to the efficient bactericidal action of complement.

Composition of the growth medium has also been shown to affect the serum sensitivity of bacterial cultures. A strain of Escherichia coli was demonstrated to be more susceptible to serum killing when grown in a simple salts medium than in a medium which was nutritionally complex, even when the growth rates were identical (Melching and Vas, 1971). The authors postulated that the difference might be related to a greater ability of the cells grown in the complex medium to repair complement-mediated damage to their membranes. Similarly, Salmonella typhimurium grown in diluted broth was found to be more sensitive than when it was grown in undiluted broth to killing by human serum (Maaløe, 1948).

Because bacterial species demonstrate a wide array of biochemical and physiological requirements, there is no widely accepted standard assay for serum bactericidal activity. Thus, it is important for investigators to standardize conditions used in their experiments and to explicitly describe their methods when reporting results. Serum sensitivity measurements are generally made by incubating organisms

with a suitable concentration of serum, with or without the addition of an exogenous complement source, and determining the change in the number of live organisms by a colony counting technique after certain time intervals. Viable colony counting techniques are well known for producing results with high standard deviations, leading investigators to adopt multiple sampling methods which are time consuming and laborious. Muschel and Treffers (1956) thus developed an alternative assay based on an increase in optical density due to regrowth of surviving organisms after their removal from the conditions of the bactericidal assay. However, a comparison of this method with a conventional plating technique revealed a consistently lower number of survivors as determined by the former than by the latter method, suggesting that a temporary lag in cell division occurred in the photometric assay (Michael and Braun, 1964). Other rapid assay systems have been developed, including a plaque assay which involves spotting serum dilutions on a plate streaked for confluent growth (Fierer et al., 1972), an assay based on loss of ability to synthesize protein (Kato and Bito, 1978), a radioassay to determine release of ^{51}Cr-labelled lipopolysaccharide (Fierer et al., 1974), and a radioactively-labelled DNA release method (Friedlander, 1975).

Phagocytosis and Intracellular Killing of Bacteria by Phagocytes

Metchnikoff (1887) was the first to publish the theory that bacteria could be destroyed within a host by phagocytes. The prevailing theory at that time was that resistance to bacterial infections depended entirely upon humoral factors in the serum, leading to a major controversy

between the humoral versus cellular theories of immunity (Wood, 1960). The two factions were somewhat reconciled by the discovery that antibodies and phagocytes could work together in destroying bacteria. Wright and Douglas (1903) called the serum factors that assisted in phagocytosis "opsonins" from the Greek meaning "to prepare for dining." Later investigations revealed that fresh normal or preimmune serum has opsonic power that could be destroyed by heat, whereas serum from immunized animals contained heat-stable opsonic factors (Mudd et al., 1934). In addition, it was shown that in some instances, the heat-labile and heat-stable serum factors could work cooperatively (Ward and Enders, 1933).

The two types of phagocytes that defend against microbial infections are polymorphonuclear leukocytes (PMN) and mononuclear phagocytes (peripheral blood monocytes and tissue macrophages). PMN are thought to be primarily responsible for defense against extracellular, invasive organisms which tend to cause acute infections. Mononuclear phagocytes, on the other hand, are more important in defense against facultative or obligate intracellular organisms which tend to cause chronic infections (Cline and Territo, 1980). Organisms that resist intracellular killing by phagocytes can actually be transported in them to sites distant from the primary focus of infection. Mononuclear phagocytes generally respond to more lightly opsonized bacteria than do PMN (Stossel, 1975). However, they are less efficient than PMN at killing conventional microorganisms (Densen and Mandell, 1980).

The first step in the phagocytic process involves formation of a stable bond between the phagocyte and the organism. This may be accomplished inefficiently by chance contact in a fluid medium or

somewhat more efficiently by what Wood (1960) termed "surface phagocytosis." This term refers to the ability of phagocytes to trap organisms against the surfaces of tissues or against each other's surfaces when the cells are packed closely together in thick exudates. Opsonization of bacteria by antibody provides a more efficient method of bond formation. Antibody can act as a bifunctional molecule which binds to bacteria by its Fab region and then activates ingestion through the Fc region (Stossel, 1975). This action is implied by the observation that papain- or pepsin-digested antibody, containing Fab fragments only, will bind to bacteria but does not opsonize them (Quie et al., 1968). Microorganisms may also be opsonized by the sequential interaction of antibody and the classical components of complement: C1, C4, C2 and then C3; or by deposition of C3b on the organism through the alternate complement pathway in the absence of antibody (Stossel, 1975). Opsonization thus involves deposition of antibody and/or complement on the surface of an organism, leaving the Fc fragments of the antibody molecules and the C3b fragments of complement exposed to react with specific plasma membrane receptors found on phagocytes (Wilkinson et al., 1979a). Polymorphonuclear leukocytes contain receptors only for the Fc portion of IgG, so opsonization of bacteria by other classes of immunoglobulin does not promote ingestion by those cells (Densen and Mandell, 1980).

Opsonization also is thought to have another mode of action. Normally, both phagocytes and organisms have a negative surface charge which leads to an electrostatic repulsion between the two cell types. Opsonization results in an increased hydrophobicity of organisms and a

reduction in the charge repulsion between them and adjacent phagocytes (van Oss, 1978; Winter, 1979).

Once a bond between the phagocyte and the organism has been established, the actual process of phagocytosis begins. Adherence stimulates local cell membrane activity so that a pseudopod is formed which flows around the organism, engulfs it, and then fuses to leave the particle enclosed in a membrane-lined vacuole, a phagosome. Griffin and coworkers (1976) demonstrated with macrophages that opsonic binding of phagocytes to particles was not necessarily followed by ingestion. They found that ingestion required homogeneous distribution of opsonins over the entire surface of the particle as well as receptors over the surface of the macrophage membrane. The cellular events occurring after ingestion which lead to intracellular killing and degradation of phagocytized organisms have been reviewed recently (Elsbach, 1980), and will not be included here.

Intact organisms are generally not susceptible to the action of degradative enzymes; therefore digestion of engulfed organisms is, to a large extent, determined by the cell envelope protecting the organisms and the ability of the phagocyte to degrade or penetrate the envelope. Many gram positive bacteria with relatively simple envelopes are more easily and more rapidly degraded than gram negative organisms that are protected by an envelope containing an additional outer membrane (Elsbach, 1980). Escherichia coli has been shown to become more sensitive to lysosomal degradation after boiling (Cohn, 1963) and after induction of spheroplast formation (Patriarca et al., 1972).

Bacterial degradation does not necessarily have to occur for killing of the organisms to take place. PMN have been shown to kill > 95% of an

E. coli population (as determined by an inability to multiply) within 15 min after ingestion. The organisms did not, however, show major structural and functional disorganization (Elsbach, 1980). Nevertheless, ingested E. coli rapidly develop increased membrane permeability, as demonstrated by entry of Actinomycin D into bacteria which would normally be impermeable to that antibiotic (Leive, 1974).

Testing of phagocytic capacity in vitro requires attention to details of cultural conditions much like the testing of serum bactericidal activity mentioned above. Pseudomonas cepacia grown in batch culture under conditions of specific nutrient depletions showed varying degrees of sensitivity to engulfment and killing by human PMN (Anwar et al., 1983). Similarly, Finch and Brown (1978) used chemostat-grown organisms and reported that slow-growing magnesium-limited cells of Pseudomonas aeruginosa were significantly more resistant to the lethal effects of rabbit PMN than were either fast-growing magnesium-limited cells or slow-growing carbon-limited cells. Those investigators hypothesized that the differences in sensitivity of organisms grown under various conditions reflect phenotypic variations in their cell envelope composition. Such variations could lead to changes in the surface free energy of the cells or alterations in sensitivity to opsonization.

The growth phase of the organisms tested also has been shown to have an effect on phagocytosis of microorganisms. Mezzatesta and Rest (1983) found that human blood monocytes were able to kill stationary-phase Neisseria gonorrhoeae but could not kill log-phase organisms. Whitnack et al. (1981), utilizing a system in which binding of organisms to macrophages could occur but internalization could not, found that

attachment of early log-phase Group A Streptococcus was low, whereas attachment was high for organisms in late log phase and thereafter.

Phagocytosis and killing as measured *in vitro* may depend on the incubation temperature used. An increased bactericidal capacity of human PMN incubated at 40° C compared with those maintained at 37° C was observed with *E. coli*, *S. typhimurium*, and *Listeria monocytogenes*, but not with *Staphylococcus aureus* (Mandell, 1975; Roberts and Steigbigel, 1977). Anwar et al. (1983) similarly found that exponential-phase cells of *P. cepacia* exposed to human PMN had a consistent decrease in survival as the incubation temperature of the phagocytes was increased from 33° C to 41° C.

The earliest studies of phagocytosis were performed by light microscopic examination of fixed smears of phagocytes after ingestion of bacteria. It was realized, however, that it is often difficult to differentiate between non-specific attachment of organisms to phagocytes and actual internalization of bacteria (Mudd, 1934; Finch and Brown, 1978). Techniques in electron microscopy have also been used to demonstrate internalization of organisms and other cells within membrane-bound vacuoles of phagocytes (Chambers, 1973; Maheswaran et al., 1980).

Indirect assays of phagocytosis utilize indicators such as reduction in viability of phagocytized organisms or metabolic stimulation of phagocytes after ingestion of organisms. The bactericidal assay measures a decrease, over time, in the viable count of a mixture of phagocytes and bacteria; interpretation of results assumes that phagocytized organisms have been killed. The limitations of this approach include its laboriousness and its inability to discriminate

between changes in viable counts due to phagocytosis and those due to other factors. Growth of unphagocytized organisms or agglutination of bacteria, which may occur over the relatively long incubation times required, can lead to misinterpretation of results (Stossel, 1975). The use of metabolic indicators of ingestion by phagocytes, such as the rate of glucose oxidation to CO_2 or reduction of nitroblue tetrazolium, has an advantage of allowing measurement without prior separation of the phagocytes from unphagocytized organisms. However, the metabolic activity of resting cells contributes background activity, and increased cellular metabolism has been shown to be stimulated by the components of certain organisms without actual particle uptake (Cohn and Morse, 1960).

Perhaps the most theoretically sound methods for assay of ingestion involve direct determination of the number of organisms within phagocytes. This may be done by radioactively labelling the organisms and measuring phagocyte-associated radioactivity (Verhoef et al., 1977; Hof et al., 1980) or by determining the number of viable organisms within phagocytes after short incubation periods (van Furth and van Zwet, 1973; Rush et al., 1981). The major technical problem in conducting these assays is that extracellular organisms must be removed from the reaction mixture before measurement of cell-associated organisms is made. Separation is usually accomplished by extensive washing of phagocytes which have been attached to glass or plastic surfaces in monolayers (Campbell et al., 1983) or by differential centrifugation of cells and bacteria in suspension (Verhoef et al., 1977; Rush et al., 1981). Neither of these methods can adequately remove organisms that are bound to the surface of phagocytes but are not actually internalized.

When both intracellular killing and phagocytosis are to be measured, viable counting techniques are generally used. An additional problem encountered with these techniques is growth of unphagocytized organisms. Multiplication of extracellular organisms can invalidate mathematical calculations of phagocytosis made from viable counts of supernatant samples, and also can be misinterpreted in intracellular killing assays as intracellular multiplication. To circumvent these problems, several investigators have added antibiotics to suspensions of phagocytes after separation from unphagocytized organisms (Kapral and Shayegani, 1959; Alexander and Good, 1968; Hsu and Radcliffe, 1968). Other groups have argued that antibiotics can penetrate phagocytes (Cole and Brostoff, 1975). Rhodes and Hsu (1974) therefore incorporated antiserum instead of antibiotics to control the growth of extracellular organisms. An additional method for determining intracellular killing has been developed, based on the assumption that release of DNA from organisms can be taken as direct evidence of cell death (Friedlander, 1978; Davies, 1982). In this method, the amount of radioactivity released from organisms that have been phagocytized is measured. Measurement by this technique is not obscured by growth of extracellular organisms and is absolutely specific for organism death, but it may lack sensitivity for certain organisms that resist the extensive degradation necessary for bacterial DNA to be released.

Roles of Capsular Material in the Pathogenesis of Selected Organisms

Bacterial exopolysaccharides are a complex group of polymers which are generally formed from repeating units of 2-6 monosaccharide residues

assembled by membrane-bound enzymes and are then extruded from the cell surface (Sutherland, 1972). They may then form a slime layer which is apparently unattached to the bacterial cell or a discrete capsule attached by some unknown mechanism to the outer layer of the cell wall. The fact that nonmucoid variants of normally encapsulated organisms are frequently found in the laboratory plus the knowledge that capsules can be removed physically or enzymatically without killing the organisms, would indicate that exopolysaccharides are not essential for the growth and survival of the bacteria on which they are found. However, other observations point to their importance to the organisms. Certain bacteria, such as Klebsiella aerogenes and Streptococcus pneumoniae, are almost always mucoid when isolated from a host animal. They often will become nonmucoid after repeated transfer in artificial media in which the nonmucoid form grows as well or better. This suggests that some factor in the normal host environment favors the selection of mucoid variants (Wilkinson, 1958).

The association found in many pathogenic organisms between degree of encapsulation and increased virulence is another indication that capsular material is of value to certain organisms (Smith, 1977; Densen and Mandell, 1980; Tajima et al., 1982). Pathogenicity refers to the ability of microorganisms to produce disease in a host. As stated by Smith (1977, p. 476), "Whether, in a particular instance, disease occurs or is prevented depends on the outcome of interactions between the microbe and the host." The majority of such interactions involve the surface of the organism with host defense mechanisms, including adherence of the organism to host epithelial surfaces, degree of microbial resistance to humoral antibodies and other antimicrobial

substances, and degree of resistance to ingestion and intracellular killing by host phagocytes. Thus any surface component, including capsular material, which renders an organism more resistant to host defenses must be considered as a potentiator of pathogenicity, and thus a virulence factor.

Specific interactions between surface components of bacteria and host mucosal surfaces are responsible for determining which organisms, whether pathogens or commensals, will selectively adhere to certain host surfaces. For example, the K88 capsular antigen on the surface of E. coli strains that are enteropathogenic for piglets is considered to be a virulence factor because it determines the ability of the organisms to attach to the brush border of the upper small intestine (Smith, 1977). E. coli strains lacking the K88 antigen do not attach and are avirulent. By the same token, piglets genetically deficient in the epithelial receptor for the K88 antigen are naturally resistant to the disease. Capsular material of Mycoplasma gallisepticum, a cause of respiratory disease in chickens and turkeys, has also been implicated as a virulence factor because of its association with the adhesive properties of the organism to avian tracheal epithelial cells (Tajima et al., 1982).

The presence of capsular material on the surface of certain bacteria has been shown to render the organisms resistant to agglutination by normal serum and by serum containing antibodies specific for somatic O antigens. Muschel et al. (1958) found a marked correlation between the capsular content of several Salmonella typhi strains and their inagglutinability with anti-O antiserum. Extracts of capsular material from gram negative bacteria have also been shown to inhibit agglutination in serologically unrelated systems. For example, extracts

of capsular K antigens from E. coli were shown to quantitatively inhibit agglutination of sheep erythrocytes by rabbit anti-sheep serum (Glynn and Howard, 1970). Ceppellini and Landy (1963) suggested that the ability of K antigens to suppress agglutination was related to their viscosity. Others have related it to their large negative charge (Wilkinson, 1958), or to inhibition of lattice formation between adjacent bacteria (Glynn and Howard, 1970).

Capsular material has also been shown to protect certain gram negative bacteria from the bactericidal effects of serum. The capsular K antigen of K. aerogenes, an opportunistic pathogen of debilitated humans, was found to be involved in determining the resistance of the organism to complement-mediated serum killing (Williams et al., 1983). Strains of encapsulated organisms were resistant to normal human serum but nonencapsulated organisms were serum sensitive. Furthermore, resistant strains are frequently isolated as the causative agents of infections involving tissue damage, leading to the suggestion that serum resistance is an important determinant of virulence in at least some infections due to gram negative bacteria (Taylor, 1983). Chelation of the serum with Mg-EGTA to prevent activation of the classical complement pathway delayed the rapid serum killing of non-encapsulated organisms by approximately 15 min. In another system, fresh preimmune rabbit serum without demonstrable antibodies was bactericidal for two nonencapsulated strains of Haemophilus influenzae, whereas two encapsulated strains were resistant to the same serum (Branefors and Dahlberg, 1980). Heat treatment of the serum at 56° C for 5 min destroyed the bactericidal effect. Hyperimmune rabbit serum was bactericidal for both encapsulated and nonencapsulated strains,

even after heating at 56° C for 30 min. It was proposed by those investigators that encapsulated H. influenzae lacked the ability to activate complement through the alternate pathway, even though they were very sensitive to complement activated through the classical pathway. Capsular K antigens have also been shown to influence the sensitivity of E. coli strains to complement-mediated killing (Glynn and Howard, 1970). Complement resistance in 4 strains was proportional to their K antigen content as measured by immunodiffusion. In addition, the strains became more sensitive to complement when the organisms were grown at non-optimal temperatures at which a reduced amount of K antigen was produced.

The mechanism by which the presence of capsular substances render organisms more resistant to serum bactericidal effects is not clearly understood. Surface structures have been thought to cause activation of complement at so great a distance from the cytoplasmic membrane that the intermediates decay before they can be incorporated into functional membrane attack complexes (Rowley, 1973). It has also been suggested that capsular material and long O side chains on lipopolysaccharide of smooth organisms may impede antibody binding and subsequent attachment of complement components to the surface of the organisms (Glynn and Howard, 1970). However, other evidence suggests that polysaccharide capsules do not act as diffusion or permeability barriers to macromolecules such as IgG, certain complement components, and lysostaphin (King et al., 1980; King and Wilkinson, 1981). Still other evidence exists which would indicate that capsular material does not necessarily confer serum resistance on organisms. For instance, most Klebsiella strains produce abundant capsular material, but many of them are also

susceptible to the bactericidal effects of serum (Fierer et al., 1972). In addition, the isolates of P. aeruginosa recovered from patients with cystic fibrosis are generally extremely mucoid, but they also are frequently more susceptible to serum killing than are strains of the organism isolated from other sources (Taylor, 1983). Such epidemiological comparisons make it very difficult to propose a general mechanism for the role of capsular material in serum resistance of gram negative organisms.

Perhaps the most important function of capsular material in the pathogenesis of bacteria is that of protection against phagocytosis. As stated by Wilkinson (1958, p. 66), "Although there are probably many factors which cause resistance of a microorganism to phagocytosis, it is clear that one of the most important is the presence of a capsular polysaccharide." Among the organisms possessing capsules that prevent ingestion by phagocytes are Streptococcus pneumoniae, Streptococcus pyogenes, Neisseria gonorrhoeae, Neisseria meningitidis, K. pneumoniae, S. aureus, H. influenzea Group B, E. coli, Yersinia pestis, Bacillus anthrasis, Campylobacter fetus, Bacteroides fragilis, and P. multocida (Densen and Mandell, 1980). A clear correlation between capsule-induced resistance to phagocytosis and virulence is shown with Yersinia pestis which only acquires capsules after it has infected a host (Densen and Mandell, 1980). Those organisms are transmitted by fleas and exist in the flea in a nonencapsulated state. When the organisms are transferred through the flea bite to a mammalian host, they are rapidly phagocytized and killed by PMN. Those phagocytized by monocytes, however, remain viable, multiply within the monocytes, and acquire capsules. Upon death of the monocytes, encapsulated organisms are

released that are resistant to phagocytosis by both neutrophils and monocytes. They continue to multiply, producing the symptoms of plague (Cavanaugh and Randall, 1959).

There are many other examples of organisms which are more resistant to phagocytosis when encapsulated than when nonencapsulated because of mutations or unfavorable growth conditions. Encapsulated organisms have also been shown to be more resistant to phagocytosis than those decapsulated by chemical or physical means. Many of the earliest studies of encapsulated organisms were performed with the pneumococcus with which a clear correlation was found between the mucoid nature of colony (indicating degree of encapsulation), virulence, and resistance to phagocytosis (Wilkinson, 1958). Fresh, encapsulated clinical isolates of S. aureus also were found to be more resistant to phagocytosis by human PMN than were the same strains after repeated subculturing on artificial media, whereby they reverted to nonencapsulated types (Karakawa and Young, 1979). The laboratory-propagated strains also were found to be less virulent for mice than were the parent strains. The same investigators found that phagocytosis of nonencapsulated strains by PMN occurred readily in the presence of either normal or immune serum, whereas phagocytosis of encapsulated strains required the presence of specific homologous antiserum. Encapsulated strains of E. coli also were found to be more resistant than nonencapsulated strains to phagocytosis and killing by bovine PMN, requiring approximately 100 times more serum opsonins that the nonencapsulated strains (Hill et al., 1983). Williams et al. (1983) studied another gram negative organism, K. aerogenes. They found that in the absence of specific antiserum, two encapsulated strains were not ingested to any appreciable

extent by PMN in whole human blood. Addition of homologous anti-capsular serum to the bacteria resulted in a dramatic increase in phagocytic ingestion. Antiserum made to a smooth but nonencapsulated strain (anti-O serum) also effectively opsonized one of the encapsulated strains, but it failed to opsonize the second encapsulated strain unless the amount of capsular material surrounding the cell was physically reduced.

Although it has been agreed for many years that the presence of capsular material causes bacteria to be more resistant to phagocytosis, the mechanism for such resistance has not been clearly understood. In 1945, Dubos stated that encapsulated pneumococci maintained a definite distance from leukocytes by a sort of negative chemotactic effect, presumed to be due to a repulsion between the hydrophilic capsule and the hydrophobic phagocyte surface, or to a repulsion between the negative charges on the two cell surfaces (Wilkinson, 1958). It was thought, then, that anti-capsular antibodies opsonized by combining with the surface layer of the capsule, thereby reducing its hydrophobic character and its negative charge. However, other investigators of the time produced evidence to the contrary. Rothbard (1948) found that a group A streptococcus, which possessed a hyaluronic acid capsule impregnated with a protein component called M protein, was a million times more virulent than the corresponding variants possessing capsules without M protein. The M protein should, according to the prevailing theory, have acted much like an antibody, reducing the negative charge and hydrophilicity of the capsule and making the organisms more sensitive to phagocytosis and thus less virulent.

In other attempts to elucidate the mechanisms of capsule-induced resistance to phagocytosis, the size of capsules was correlated with degree of resistance conferred. Wood and Smith (1949) found that the high resistance to surface phagocytosis of type 3 S. pneumoniae compared to other pneumococcal types, was due to the possession of a larger capsule. Another group of investigators working with the same organism reported that the different susceptibilities to phagocytosis among strains was due to differences in the chemical composition of the capsules (MacLeod and Krauss, 1950). However, they also reported that resistance by mutants of a given capsular type correlated directly with the amount of capsular material each produced. Similarly, Melly et al. (1974) demonstrated a direct correlation between the capsule size of S. aureus strain M and its resistance to phagocytosis by PMN. Still, factors other than capsule size have to be important because group A streptococci which had been decapsulated with hyaluronidase, were still found to be engulfed only slowly by leukocytes and to be highly virulent (Rothbard, 1948). Such inconsistencies led Wilkinson (1958, p. 67), to state, "It is also possible that an important factor in governing phagocytosis is the sensitization of the bacterial surface by a substance present in nonimmune serum. Extracellular polysaccarides may prevent this combination."

Within the last 10 years, the importance of the "substance present in nonimmune serum" that Wilkinson alluded to has been shown to be complement. Complement is deposited on the surface of bacteria after activation of the classical pathway by antibody deposition on the organism, or by the alternate pathway in the absence of antibody (see section on Phagocytosis). Most organisms are known to be opsonized, as

long as they are not in an encapsulated state, by complement alone or by complement plus antibodies found in many normal sera to their major cell wall components (Densen and Mandell, 1980). There are two possible explanations for the failure of encapsulated organisms to be opsonized by normal serum or by serum with antibodies to cell wall, but not capsular antigens. Capsular material could either block the binding of opsonins to the organism or mask opsonins that are bound to the surface of an organism, preventing the opsonins from acting as ligands between the organism and the phagocyte. Experimental evidence has since been obtained which supports both of these hypotheses.

In a study supporting the hypothesis that capsular material blocks binding of opsonins to the surface of the organism, Stinson and van Oss (1971) performed adsorption studies with ^{125}I-labelled IgG and S. aureus. They found that encapsulated organisms adsorbed 70% more protein than did heat-treated, decapsulated organisms, and more than twice as much as did a nonencapsulated strain. They felt these results suggested that a certain quantity of the IgG was physically trapped within the capsule matrix. Horwitz and Silverstein (1980) also presented evidence supporting this theory. They found that PMN phagocytized and killed encapsulated E. coli only in the presence of complement and antibacterial antibody, whereas they phagocytized and killed a non-encapsulated mutant of the same strain in the present of complement alone. They further demonstrated by fluorescence microscopy that antibody had to be present for encapsulated, but not nonencapsulated, organisms to fix complement to their surface. They concluded that in the absence of antibody, the E. coli capsule blocks complement fixation to the bacterial surface, probably by covering surface lipopolysaccharide

capable of activating complement in the absence of antibody. In another study in which 21 strains of several bacterial species were used, Verbrugh et al. (1979) used a quantitative fluorescent immunoassay to measure the amount of the C3 component of complement bound to the surface of bacteria. They found that encapsulated strains of S. aureus, E. coli, K. pneumoniae, and S. typhi failed to bind significant amounts of C3 in nonimmune human serum and were also inefficiently phagocytized by human PMN. Nonencapsulated strains of E. coli and S. aureus were found to fix C3 readily and to be efficiently phagocytized. They concluded that "encapsulation interferes with the process of complement-dependent opsonization due to masking by the capsule of other, complement-activating, cell wall components (Verbrugh et al., 1979, p. 813).

Other studies have presented evidence which supports the theory that capsular material prevents phagocytosis by masking bound opsonins to make them ineffective. Peterson et al. (1978) showed that encapsulated and nonencapsulated strains of S. aureus were virtually identical in their abilities to activate complement in nonimmune human serum, as determined by a complement consumption assay. The same group (Wilkinson et al., 1979b) later used electron microscopy and peroxidase-labelled anti-C3 IgG to show that C3 was localized on the cell wall of both encapsulated and nonencapsulated S. aureus, implying that the capsule does not act as a barrier to either the early complement components or to IgG molecules. They also showed that in the presence of non-immune serum, C3 was found beneath the capsular polysaccharide and the organisms were not effectively opsonized. In the presence of type-specific immune serum, however, anti-capsular antibodies were found to be distributed throughout the capsule and the organisms were opsonized.

They suggested, then, that the mechanism of resistance to phagocytosis by the encapsulated strain was related to the physical masking of bound C3 molecules by the capsule, preventing the interaction of activated C3 (C3b) with receptors in the PMN plasma membrane. They further suggested that when anti-capsular antibodies are present, phagocytosis is mediated via their Fc fragments which are located on the capsule surface.

Perhaps a general mechanism for resistance of encapsulated organisms to phagocytosis cannot be constructed, at least within the current framework of knowledge. As stated in a recent review of the subject,

> Either or both of these mechanisms [the two presented above] may account for microbial resistance to phagocytosis, depending on the organism being studied, the nature of the opsonin (complement or antibody), and possibly, the type of phagocytic cell (Densen and Mandell, 1980, p. 825).

A final possible role of capsular material in the pathogenesis of bacteria was proposed by Schwab (1975) who reported that the capsular material of K. pneumoniae was immunosuppressive in mice. Intramuscular injection of Klebsiella polysaccharide 3 to 30 days before an injection of bovine serum albumin resulted in depression of the normal antibody response to the albumin. He considered the response to be a type of immunological paralysis.

Statement of the Research Problem

It has been known for many years that capsular material exists on Pasteurella haemolytica. However, little is known about its involvement in pathogenicity of the organism. Before an assessment of the role of capsular material on a bacterium can be made, one must first demonstrate its presence and then produce organisms without it. Comparisons then

can be made between bacteria which are encapsulated and those which are not. The objectives of this study were: to develop a rapid and reliable capsular staining technique and a capsular extraction procedure which would yield live cultures of decapsulated bacteria; to compare the resistance of encapsulated and decapsulated organisms to the agglutinating and bactericidal effects of sera from immune and nonimmune cattle; to compare the resistance of encapsulated and decapsulated organisms to phagocytosis by and toxicity for bovine phagocytes; and to determine the general biochemical nature of the capsular extract and evaluate its immunologic and toxicologic relationship to P. haemolytica cytotoxin.

CHAPTER II

THE DEMONSTRATION OF AGE-DEPENDENT CAPSULAR MATERIAL ON *PASTEURELLA HAEMOLYTICA*

Introduction

Pasteurella haemolytica serotype 1 is the major cause of the severe fibrinous pneumonia that occurs in shipping fever of cattle (Rehmtulla and Thomson, 1981). As early as 1956, the presence of soluble capsular material on *P. haemolytica* was demonstrated and its polysaccharide nature was established (Carter, 1956a). Capsular material or diffusable surface antigens have been used subsequently to determine the serotype of this organism (Biberstein et al., 1960; Carter, 1956; Frank and Wessman, 1978). Recently, material, presumably capsular, has been extracted from *P. haemolytica* by several techniques (Evans and Wells, 1979; Gentry et al., 1982; Tadayan and Lauerman, 1981). Vaccination of mice, hamsters (Mukker, 1977; Tadayan and Lauerman, 1981), and sheep (Gilmour et al., 1979; Wells et al., 1979) with these extracts has been shown to protect them against experimentally induced pasteurellosis.

In its description of *P. haemolytica*, Bergey's Manual states "capsular material not usually demonstrated by microscopical methods" (Smith, 1974). Capsules on *P. haemolytica* from sheep have reportedly been demonstrated, and quantitative differences have been described between smooth and rough cell types (Biberstein et al., 1958). However,

we have been unable to find any publications which include photographs of encapsulated organisms.

The purposes of the studies reported herein were to demonstrate the capsule on P. haemolytica serotype 1 and to examine the effect of culture age on capsular integrity. Studies with other serotypes of P. haemolytica were not performed.

Materials and Methods

Culture Media

Two solid types of media were used for culturing the organisms. The first was a modified brain heart infusion agar (Difco Laboratories, Detroit, MI) which contained 5% bovine blood, 10% horse serum (M.A. Bioproducts, Walkersville, MD), and 1% yeast hydrolysate (ICN Pharmaceuticals, Cleveland, OH). In addition, a modification of Sawata's medium (Kume et al., 1978) with a brain heart infusion base was used. A corresponding Sawata's broth was also used.

Microorganism

The organism used throughout the study was P. haemolytica serotype 1 (Frank and Wessman, 1978), originally isolated from a feedlot calf. The organism was maintained in the lyophilized state. For each experiment, a lyophilized culture was reconstituted with distilled water and streaked for isolation onto appropriate media. After 22 hr of incubation at 37° C in a candle jar, isolated colonies were suspended in 0.01 M phosphate buffered saline (PBS) (pH 7.2), and the resulting suspension was used to inoculate fresh media. Cultures on solid media

were incubated for various times at 37° C in a candle jar. Broth cultures were incubated at 37° C in a shaking water bath.

Fluorescent-Antibody Technique (FAT)

Antiserum to P. haemolytica serotype 1 was made by injecting chickens intravenously with whole cells at four 1-week intervals as described previously (Gentry et al., 1982). Pooled antisera were then conjugated with fluorescein isothiocyanate (BBL Microbiology Systems, Cockysville, MD) (Corstvet and Sadler, 1964). At 2-hr intervals after inoculation (up to 22 hr), thin smears of water-suspended organisms were made on glass coverslips and allowed to air dry. Smears were subsequently fixed in acetone for 10 min and stained with conjugated antiserum for 25 min at 37° C. A 1:5 dilution of antiserum was found to be optimal. The coverslips were then washed in 3 changes of PBS for a total of 20 min, mounted on glass microscope slides with a 90% PBS-10% glycerol mounting medium (pH 7.2), and examined microscopically with an ultraviolet light source.

Capsular Staining Techniques

Maneval's method (Maneval, 1941). One drop of PBS-suspended organisms was mixed on a glass microscope slide with an equal drop of 1% aqueous Congo red stain (Allied Chemical, New York, NY). The mixture was spread into a thin film smear and allowed to air dry. Without fixation, the smears were counterstained with Maneval's stain for 2 min, drained, and blotted dry.

India ink method (Butt et al., 1936). Bacterial growth was suspended in sterile 6% glucose on a glass microscope slide. This

suspension was then mixed with a drop of waterproof ink (Pelikan, Hannover, Germany). The mixture was spread into a thin film smear and allowed to air dry. The smear was fixed for 1 min in absolute methanol, rinsed in water, counterstained with 1% aqueous crystal violet (Difco Laboratories, Detroit, MI) for 1 min, and rinsed in water.

Jasmin's method (Jasmin, 1945). Bacterial growth was suspended on a microscope slide in PBS containing 0.75% phenol and 10% horse serum. A thin film smear was made from the suspension and allowed to air dry. The smear was fixed by rapid immersion in absolute methanol followed by flaming. It was counterstained with 1% aqueous crystal violet for 1 min and rinsed in water.

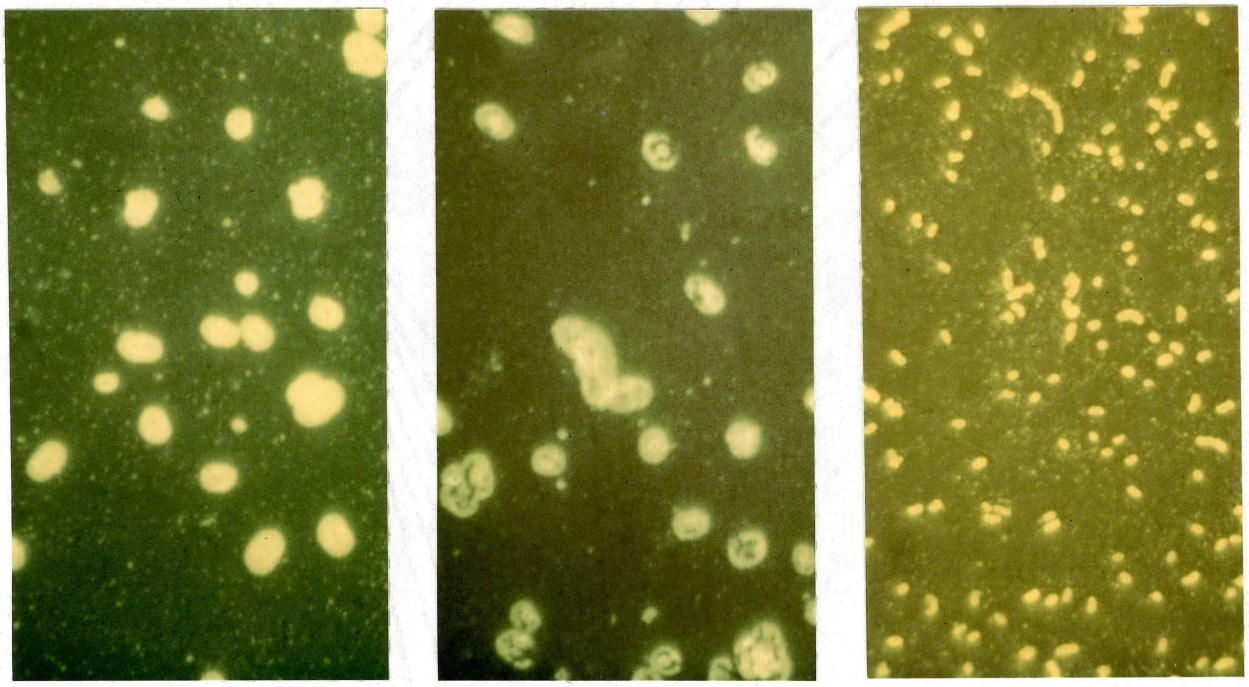
Other methods. The eosin-serum and Hiss methods were performed as described previously (Hiss, 1905; Howie and Kirkpatrick, 1934).

Smears stained by each technique were examined under a 100x oil immersion lens of a light microscope.

Results

Smears prepared by the FAT showed very bright fluorescent staining of the cell walls. Organisms from cultures incubated for less than 12 hr were surrounded by wide capsules that stained less intensely than the cell walls (Figure 2.1a). Capsules appeared to be widest around organisms from 2-hr cultures. After 12 hr of incubation, the capsular material had a mottled appearance and only partially encircled the cells (Figure 2.1b). In smears prepared from cultures 16 hr or older, capsular material usually could not be demonstrated (Figure 2.1c).

Figure 2.1. Demonstration of Age-Dependent Capsular Material with a Fluorescent Antibody Stain. P. haemolytica stained with fluorescein isothiocyanate-conjugated antiserum (x 500). (a) Cells from a 6-hr culture with capsules. (b) Cells from a 12-hr culture. Capsules have a mottled appearance. (c) Cells from a 22-hr culture. Capsular material is no longer apparent.



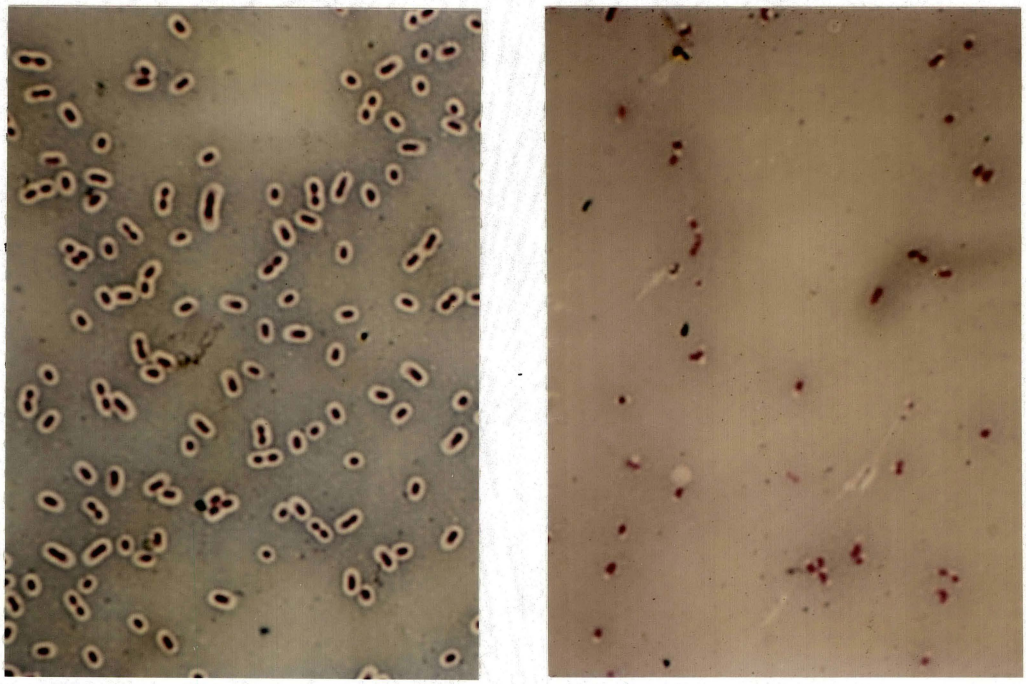
A B C

If present, the capsular material was often found only at the poles of the cells.

Capsular material also was demonstrated surrounding 6-hr cells stained by Maneval's, Jasmin's, and the India ink methods. Capsules were not demonstrated by the eosin-serum or Hiss techniques. The results of staining by the Maneval's method correlated with the FAT in that capsules were clearly and consistently demonstrated on cells from 6-hr cultures (Figure 2.2a). The bacterial cells stained red, and the background stained blue. The capsules appeared as a wide clear zone. Capsules were larger with this method than with any of the other light microscopy capsule staining techniques. The majority of the cells in a 22-hr culture appeared to have very little of the extracellular material, which again was concentrated at the poles of the cells (Figure 2.2b). The age-dependent character of capsular association with cells was demonstrated to occur on both solid media and Sawata broth.

In the Jasmin preparation, capsules appeared as narrow clear zones around the cells, but both cells and background stained purple, and there was not sufficient contrast to make the capsules obvious. Repeated efforts and modifications did not significantly improve the quality of the preparations. The India ink method stained the cells purple and the background black. The capsules again appeared as clear zones around the cells. However, the granularity and darkness of the background made it difficult at times to see unencapsulated organisms or to measure the size of the capsules around encapsulated ones.

Figure 2.2. Demonstration of Age-Dependent Capsular Material with Maneval's Stain. *P. haemolytica* stained by the Maneval method (x 1,250). (a) Cells from a 6-hr culture. Capsules appear as wide clear zones. (b) Cells from a 22-hr culture. Minimal capsular material is present on cells.



A

B

Discussion

Capsular material was demonstrated on young cultures of P. haemolytica serotype 1 by the FAT and other capsular staining techniques. The dry film India ink and Jasmin methods demonstrated capsular material, but Maneval's stain was superior to either in that it correlated with the FAT results, and the capsules appeared larger and more distinct. The Maneval's method is simple and rapid, making it excellent for following the dissolution of capsular material during extraction procedures, as well as for screening cultures or even individual colonies for capsule production.

Exopolysaccharide production by several organisms has been reported to be dependent on culture age, but those organisms differ in the growth stages in which production occurs. A mucoid strain of Pseudomonas aeruginosa was shown in batch culture to produce polysaccharide throughout the exponential growth phase, and both growth and polysaccharide production ceased simultaneously (Mian et al., 1978). For other organisms, notably Bacillus licheniformis (Troy, 1979) and Pseudomonas sp. strain MCIB 11264 (Williams and Wimpenny, 1977), capsular polysaccharide production was not concommitant with exponential growth of the organisms but was initiated in late log-phase and occurred primarily after the cultures entered stationary-phase. For type III Pneumococcus (Bukantz et al., 1941) and an organism identified as Aerobacter aerogenes (Duguid and Wilkinson, 1953), most of the total polysaccharide production occurred after cessation of logarithmic growth when cell numbers were greatest, but the rate of production per cell was highest in the early logarithmic-phase and declined progressively thereafter.

It was further reported with A. aerogenes that the average capsule diameter increased during and after the log-phase to a maximum at 48 hr (Duguid and Wilkinson, 1953).

Our results with P. haemolytica serotype 1 indicate that maximum cell-associated capsular material is produced during the early logarithmic growth phase. Capsules appeared to be widest in organisms from cultures 2 to 6 hr old and diminished gradually as the culture aged, until little capsular material remained on cells from cultures over 16 hr old. A similar age dependency of capsular association has been demonstrated ultrastructurally with Klebsiella pneumonia (Schmid et al., 1981). In that study, the majority of cells harvested in the stationary growth phase (36 hr) exhibited shrunken capsules with coarse and short filaments or no capsule at all, whereas a capsule was demonstrated on almost all bacteria harvested in the logarithmic growth phase (5 to 12 hr). In addition, the hyaluronate capsule of a type 5 strain of group A streptococcus was reported to be present on cells from early log-phase cultures but absent on cells from cultures in the later log-and stationary-phases (Whitnack et al., 1981).

The cause of the age-dependent phenomenon as demonstrated in P. haemolytica, K. pneumonia, and group A streptococcus is unknown. We have, however, observed (Gentry, unpublished data) that the cell wall ultrastructure of P. haemolytica differs topographically in organisms from 6-hr as compared to 22-hr cultures. Alterations in cell wall integrity may allow leakage of intracellular components into the surrounding capsular material, resulting in either alterations in capsular pH or enzymatic degradation of the capsule. Such degradation has been shown to occur in Bacillus licheniformis (Troy,

1973). The polyglutamic acid produced by late log-phase cells of this organism is originally bound firmly to the cell surface as a capsule. After maximum production, however, there is a fairly rapid loss of this capsular material without cell lysis due to elaboration into the culture medium of an endo-type, poly (γ-D-glutamyl) depolymerase.

In conclusion, the amount of capsular material associated with P. haemolytica serotype 1 cells is maximal in young (less than 12-hr) cultures. This indicates that to obtain capsular material in the quantities needed for chemical analysis or immunization studies, young cultures should be used.

CHAPTER III

EXTRACTION OF CAPSULAR MATERIAL FROM *PASTEURELLA HAEMOLYTICA*

Introduction

Pasteurella haemolytica has long been associated with shipping fever or pneumonic pasteurellosis of cattle. A soluble capsular substance was demonstrated when *P. haemolytica* was cultured overnight on fresh beef infusion agar plates (Carter, 1956a). Carter stated this substance was polysaccharide in nature and removed it from the organism by heating at 56° C for 30 min in water or normal saline solution. Several investigators have subsequently attempted to correlate the specificity of immunity afforded to mice by whole cell bacterins of this organism to their capsular and/or somatic antigens (Biberstein and Thompson, 1965; Knight et al., 1969).

Attempts have been made to use capsular extracts of *P. haemolytica* to confer immunity. A potassium thiocyanate (KCSN) extract of *P. haemolytica* serotype 1 cross protected mice against an intraperitoneal challenge infection of *Pasteurella multocida* type A (Mukker, 1977). KCSN extracts have also been reported to protect conventionally reared rams (Mukker et al., 1983) and calves (Yates et al., 1983) from experimentally induced pneumonic pasteurellosis. Sodium salicylate capsular extracts from *P. haemolytica* have also been utilized. The administration of a sodium salicylate extract protected

specific-pathogen-free lambs from an aerosol challenge exposure with the homologous organism (Gilmour et al., 1979, 1983). Capsular extracts continue to be pursued as possible immunizing agents in lieu of whole cell bacterins that have proved disappointing (Biberstein and Thompson, 1965; Wilkie and Norris, 1975; Tadayon and Lauerman, 1981).

Culture of P. haemolytica produce capsular material early (4-6 hr) in their growth cycle, a fact demonstrated by the direct fluorescent antibody (FAT) technique using antiserum to whole organisms (Corstvet et al., 1982a). By this technique, the capsule was further shown to disappear gradually as the culture aged so that little capsular material remained on the cells after approximately 16-22 hr of incubation. The purpose of the present study was to demonstrate the removal of capsular material from cells in the early logarithmic growth phase and to compare several methods of extraction for their efficacy and their deleterious effect on the cells.

Materials and Methods

Organisms Used and Culturing Procedure

The organism used was P. haemolytica serotype 1 (Frank and Wessman, 1978). This isolate was obtained originally from a feedlot calf and was maintained as described previously (Newman et al., 1982). Briefly, the culture was maintained in the lyophilized state and was passed periodically in the lungs of 5-8-mo-old calves, with isolation and lyophilization between each passage. For each experiment, the lyophilized culture was streaked onto modified Sawata's medium (Kume et al., 1978) and was incubated for 17-24 hr at 37° C in a candle jar. Isolated colonies were then chosen and suspended in 0.01 M phosphate

buffered saline (PBS) pH 7.2. This suspension was streaked on freshly prepared plates of modified Sawata's medium for confluent growth and was incubated for either 6 hr or 12 hr.

Preparation and Analysis of Capsular Extracts

The organisms were removed from the agar surface with a cotton swab and were suspended in water or PBS. The bacterial suspensions were incubated for 1 hr in a water bath at room temperature (RT, approx. 25° C), 37° C, 41° C, 45° C, or 56° C, followed by centrifugation at 17,000 x g for 15 min. The resulting extracts consisted of the supernatant fluids which were removed and filter sterilized through 0.2 μm filters.

Optical density (OD) measurements were taken on 1:9 dilutions of the original suspensions at 650 nm in a spectrophotometer (Spectronic 20, Bausch and Lomb) immediately before and after the extraction procedure. Viable counts were also performed on the pre- and post-extraction suspensions by the spot-plate method.

Protein determinations on the extracts were made by the BioRad procedure which utilizes protein binding to Coomassie Brilliant Blue (Bradford, 1976). Carbohydrate measurements, when included, were performed by the anthrone method (Dische, 1961) which tests for total hexoses, and by the α-naphthol reaction (Dische, 1961) which measures hexoses, pentoses, and methyl pentoses.

Antisera

All antisera used in the FA procedure, agglutination studies, and

the agar-gel diffusion tests were made by intravenous injections of 7-wk-old male Cornish cross chickens with 1 ml of whole cells, cells after extraction, or capsular extracts at weekly intervals for 4 weeks. At the time of the first inoculation, an additional 0.5 ml of the appropriate immunogen mixed with an equal volume of Freund's incomplete adjuvant was given subcutaneously. Serum was collected by exsanguination 11 days after the final injection.

Fluorescent Antibody Technique

Thin smears of bacterial suspensions were made on glass coverslips and were allowed to air dry. They were fixed for 10 min in acetone and stained for 25 min with a 1:4 dilution of fluorescein isothiocyanate conjugated chicken serum made to whole P. haemolytica cells (Corstvet and Sadler, 1964). The coverslips were then washed in 3 changes of PBS for a total of 20 min, mounted on glass microscope slides with a PBS/glycerol mounting medium (pH 7.2), and examined microscopically with an ultraviolet light source.

Serotests

Agglutination studies were performed on the pelleted cells from pre- and post-extraction suspensions of 6-hr and 22-hr cultures. The cells were suspended in a drop of antiserum and observed for 1 min for macroscopic agglutination.

The agar gel diffusion technique was used (0.9% Noble agar with 8.5% NaCl) (Heddleston et al., 1972). Capsular extracts were placed in the center well and antisera from chickens that had been given whole cells, cells after extraction, or capsular extracts were placed

in the outer wells. Plates were incubated at 37° C and were examined daily for 4 days.

Results

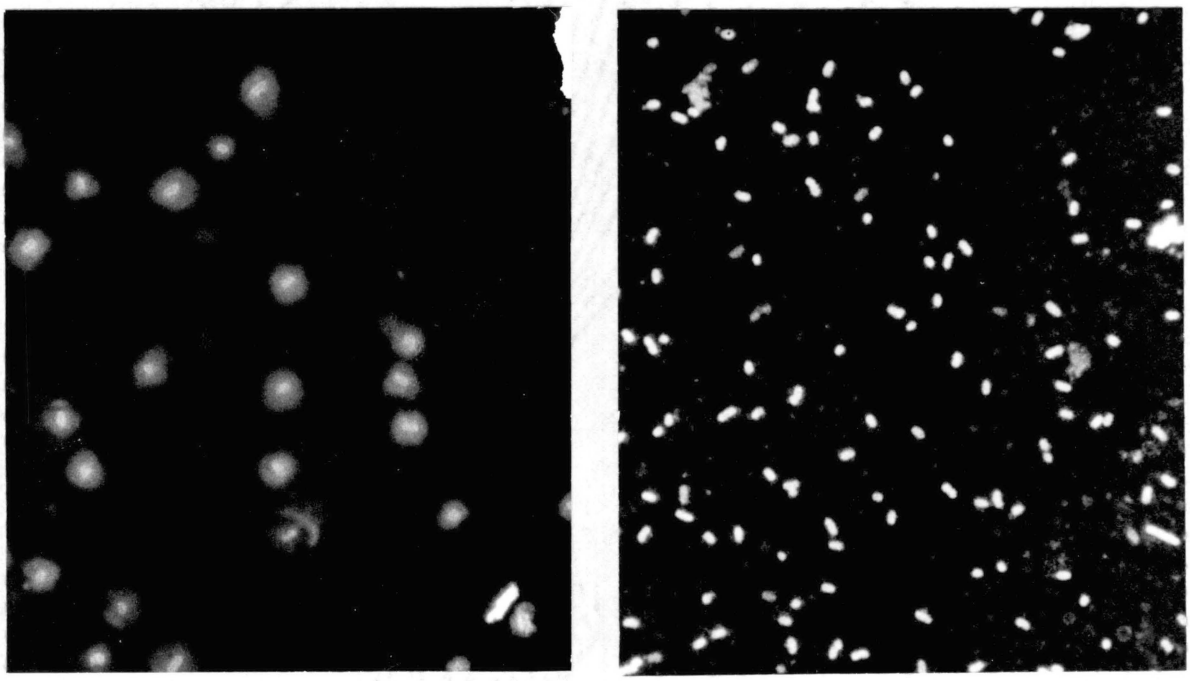
A PBS suspension of a 6-hr culture of P. haemolytica stained by the FAT showed a wide zone of capsular material around the majority of cells (Figure 3.1a). Capsular material was no longer demonstrable on cells from the same suspension after incubation at 41° C for 1 hr (Figure 3.1b).

When PBS was used as the suspending medium, an extraction temperature of 41° C was necessary to remove the capsular material from the cells, as seen by examination in the fluorescent microscope. There was little decrease in OD and cell viability in PBS suspensions at the extraction temperatures of RT, 37° C, or 41° C. It was not until the temperature of incubation was raised to 45° C that a substantial decrease in OD and viability of the suspension was observed (Table 3.1).

Capsules were also extracted from cells suspended in water. In this case, a lower incubation temperature (37° C) was sufficient to remove the capsular material (Table 3.1). However this treatment resulted in approximately a 2 log unit decrease in the viable count of the suspension. At higher temperatures, OD and viability decreased further. At room temperature, the viability decrease was low (0.1 log U), but the capsular material was not removed from the cells according to the FAT.

Protein determinations were made on extracts from various experiments (Table 3.2). Certain generalizations may be made from these data, even though numbers of cells in the suspensions varied

Figure 3.1. Demonstration with Maneval's Stain of Decapsulation of Organisms by a Capsular Extraction Technique. Cells from 6-hr cultures of P. haemolytica stained by the FA technique. (a) Pre-extraction cells. (b) Cells from the same suspension after incubation for 1 hr at 41° C in PBS x 500 oil immersion.



A B

TABLE 3.1

COMPARISON OF CAPSULAR EXTRACTION METHODS

Temperature*	Decrease in optical density∫	Decrease in viability≠	FAT Results§
H_2O Extraction:			
RT	0.03	0.1	capsules remained
37°	0.10†	1.68†	most of capsular material removed
41°	0.11	2.3	capsules removed
45°	0.23	2.2	capsules removed
56°	0.20	>6	capsules removed
PBS Extraction:			
RT	0.04	0.14	capsules remained
37°	0.03¶	0.08¶	most of capsular material remained
41°	0.06†	0.05	most of capsular material removed
45°	0.14	0.99	capsules removed
56°	0.17	>5	capsules removed

*In degrees C (RT = room temperature) of 1 hr incubation.

∫OD 650 of a 1:9 dilution of the cell suspension.

≠In log units as determined by serial dilution and spot plating.

§As determined by staining with fluorescein isothiocyanate-labeled antiserum.

†Average results from 5 trials.

¶Average results from 2 trials.

TABLE 3.2

BIORAD PROTEIN DETERMINATIONS ON CAPSULAR EXTRACTS

Extraction Temperature*	Number of Trials	Average Protein Concentration (mg/ml)
Water extracts		
RT	4	0.34
37°	7	0.53
41°	4	0.79
45°	2	0.52
56°	4	0.51
PBS extracts		
RT	1	0.11
37°	2	0.15
41°	4	0.10
45°	1	0.20
56°	1	0.22

*In degrees C (RT = room temperature).

somewhat from experiment to experiment and thus the extract protein concentrations cannot be compared absolutely. The protein content was higher for the extracts made in water than for those made in PBS. Also, in all water extraction trials in which several extracts were made from the same cell suspension at several temperatures, the protein concentration peaked at 41° C and the 45° C and 56° C extracts had a lower concentration.

Carbohydrate determinations were performed in one experiment, using a common cell suspension, and thus may be directly compared with one another (Table 3.3). The amount of carbohydrate in the various extracts tested was constant (62-78 μg/ml) regardless of the suspending medium and the extraction temperature used.

Untreated 6-hr cells with intact capsules were more difficult to agglutinate with all 6 antisera tested than were untreated 22-hr cells (Table 3.4). When 22-hr cells were treated by the extraction procedure, there was little change in agglutinability. However, when the capsules were removed from 6-hr cells, agglutination occurred more readily, and serum titers approximated those of 22-hr cells.

An agar gel diffusion plate with a 6-hr extract made in water at 37° C placed in the center well and sera from chickens inoculated with various cell suspensions or extracts as indicated in the outer wells, revealed that there was a major band missing from the reaction with antiserum made to 6-hr post-extraction cells (Figure 3.2). There were at least three separate precipitin bands in the reaction between the 6-hr extract and antiserum made to 6-hr or 22-hr whole cells (wells 1, 3, and 4; Figure 3.2). All three bands were also present when the antiserum used was made to 6-hr capsular extract

TABLE 3.3

CARBOHYDRATE DETERMINATIONS ON SELECTED CAPSULAR EXTRACTS

Extraction Temperature*	Anthrone Reaction (µg/ml)	αNaphthol Reaction (µg/ml)
H_2O Extracts:		
37°	71	62
45°	82	75
56°	90	75
PBS Extracts		
RT	79	78
41°	86	75

*In degrees C (RT = room temperature).

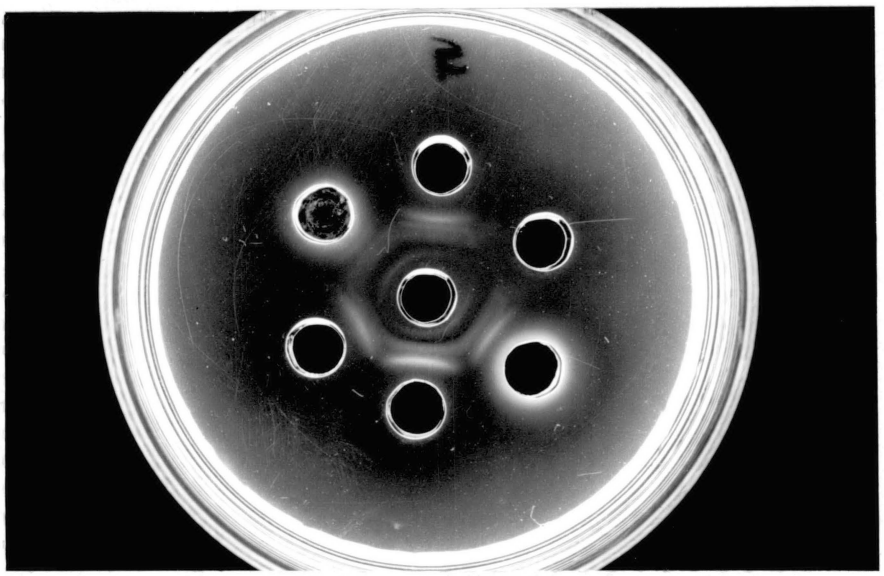
TABLE 3.4

AGGLUTINATION TITERS OF CHICKEN ANTISERA FOR ENCAPSULATED AND DECAPSULATED ORGANISMS

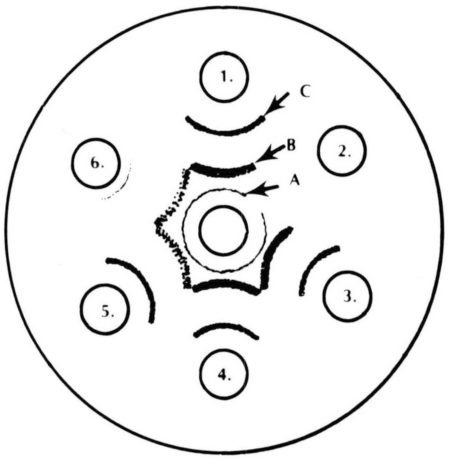
Antiserum Used*	22-hr whole cells	6-hr whole cells	6-hr extracted cells	22-hr extracted cells
6-hr whole cells	1:16	1:4	1:32	1:32
6-hr extracted cells	1:16	1:2	1:16	1:8
Extract from 6-hr cells	1:4	1:2	1:4	1:4
22-hr whole cells	1:32	1:2	1:16	1:16
22-hr extracted cells	1:16	1:1	1:16	1:16
Extract from 22-hr cells	1:4	NA	1:4	1:4

*Antisera prepared against the immunogens listed by 4 weekly intravenous 1 ml injections of 7-wk-old male Cornish cross chickens. Cell suspensions made in H_2O were extracted by incubation for 1 hr at 37° C. NA = not agglutinable.

Figure 3.2. Photograph and Schematic Representation of Agar-Gel Diffusion Plate Used to Confirm Loss of an Antigenic Component by Decapsulated Organisms. Photograph (2a) and schematic representation (2b) of agar-gel diffusion plate, using capsular extract from 6-hr cells in the center well and antisera in the outer wells from chickens inoculated with the following antigens: (1) 6-hr whole cells; (2) non-immunized control serum; (3) 22-hr whole cells; (4) 6-hr cells; (5) 6-hr extract; (6) 6-hr extracted cells. A, B, and C are precipitin lines indicating an area of antigen/antibody interaction.



A



B

(well 5), although band B was noticeably less intense. When the antiserum used was made to 6-hr extracted cells (well 6), however, not only was band B less dense, but band C was barely discernible. Normal chicken serum (well 2) did not react with the antigen.

Discussion

Cells from early logarithmic-phase cultures of P. haemolytica were encapsulated as demonstrated by the FAT. Capsular material has been shown to disappear gradually as the culture ages until it is no longer demonstrable in 16-22-hr cultures (Corstvet et al., 1982a). Concurrent with loss of capsular material, the cells are agglutinated more easily and also become osomotically fragile, as indicated by a considerable decline over a 1-hr period in OD and viable count of water suspensions of 22-hr cells (Gentry, unpublished data).

Results of the present studies show that capsular material may be removed from young cultures of P. haemolytica by a 1-hr incubation of PBS suspended cells at 41° C. With this treatment, there was little loss of cell viability, whether or not the capsular material was removed, until the extraction temperature reached 45° C. At this temperature, the lethal effect of heat itself must be considered. By contrast, the capsular material was removed from 6-hr cells suspended in water at an extraction temperature of 37° C. Concurrent with capsular removal was at least 1.5 log U decrease in the viable count of the suspensions, even at 37° C and 41° C. This loss of viability was not seen when the water-suspended cells were incubated at RT, a temperature at which the capsular material remained on the cells. It was concluded, therefore, that removal of capsular material

caused 6-hr cells to become osmotically fragile, comparable to those in a 22-hr culture, and the isotonicity of PBS provided a more protective environment, preventing cell lysis.

Freshly isolated encapsulated strains of P. haemolytica have been reported to be inagglutinable, whereas daily subculture for three weeks rendered the same organisms agglutinable (Tweed and Eddington, 1930). In the present studies, young (6-hr) encapsulated cells were more difficult to agglutinate than were those from the same culture after an additional 16 hr of incubation. After extraction, however, the 6-hr organisms agglutinated more easily, an additional indication that the capsular material had been removed.

Agar gel diffusion tests revealed that there were at least three antigens present in a 6-hr capsular extract that are also present on 6-hr whole cells. The presence of identical bands when antiserum to 22-hr whole cells was used may be explained by the fact that 22-hr cell suspensions used to stimulate antiserum production in chickens contained a small percentage of encapsulated cells, as seen by FAT. It is also possible that the 22-hr cells multiply to a limited extent in the chicken, thus presenting young encapsulated organisms to the immune system. Further, a small amount of capsular antigen from the agar plates may have been picked up by the swab and incorporated into the antigen preparation as the cell suspensions were made. Because band C was present in the reaction between 6-hr extract and antiserum to 6-hr extract or to whole cells but not when antiserum to 6-hr extracted cells was used, this band was assumed to be a capsular antigen that was removed during the extraction procedure. The faint band that did appear could reflect the failure to wash the extracted cells

before injecting them into the chickens, allowing a small amount of capsular antigen to be retained in the preparation.

The capsular extracts were shown to contain protein and carbohydrates, although it was not clearly understood how much of these components were leached from the cell interior and how much were truly of capsular origin. The amount of carbohydrate present was relatively constant (62-78 μg/ml), regardless of the extraction method used. The protein content, however, varied greatly (0.1-0.79 mg/ml). The water extracts contained 2.3 to 7.9 times more protein that did PBS extracts prepared at corresponding temperatures. These differences were thought to be due to increased cell lysis or leaching of intracellular proteins when the cells were incubated in a hypotonic environment. Capsular extracts prepared in water at 45° C and 56° C contained less protein than did extracts prepared from the same cell suspension at either 37° C or 41° C, possibly due to breakdown at the higher temperatures of protein into polypeptides too small (<3000 mol wt) to be recognized by the protein binding assay. In contrast, the protein content of PBS extracts continued to increase with increasing extraction temperature. In this case, the heat related breakdown of proteins was thought to be overshadowed by an increase in cell lysis and leakage as shown by the greater decreases in OD and viable count of PBS suspensions incubated at 45° C and 56° C than in those incubated at 41° C or less.

The extraction procedure of choice for removing capsular material with minimal concurrent cell destruction was determined to be that of incubating a PBS suspension of the organism at 41° C for 1 hr. The use of this extraction procedure permits the preparation of comparable cell suspensions with and without their capsules, such that the

pathogenicity and immunogenicity of the cultures may be investigated. Capsular extracts prepared in this way may also be studied as potential immunizing agents.

CHAPTER IV

INTERACTION OF ENCAPSULATED AND DECAPSULATED *PASTEURELLA HAEMOLYTICA* WITH BOVINE SERA

Introduction

Pasteurella haemolytica, the gram negative pleomorphic bacillus which is associated with the characteristic lesions of shipping fever in cattle, has been shown to be encapsulated, at least during the early logarithmic-phase of growth on artificial media (Corstvet et al., 1982a). Capsules also have been observed surrounding organisms in pleural fluid taken at necropsy from calves with experimental pneumonic pasteurellosis (Gentry, unpublished data). The role of the capsular material in the pathogenesis of shipping fever has not, to date, been investigated.

Bacterial capsules have long been thought to be virulence factors (Wilkinson, 1958). The presence of a well defined capsule has been shown to enhance resistance to the bactericidal effects of host serum components on many gram negative bacteria, including *Klebsiella aerogenes* (Williams et al., 1983), *Salmonella typhi* (Muschel et al., 1958), *Haemophilus influenzae* (Branefors and Dahlberg, 1980), and *Escherichia coli* (Taylor, 1975). It has been theorized that capsular material may impede binding of cell surface-specific antibody and subsequent insertion of the membrane attack complex of the complement

cascade (C5-C9) responsible for the lethal lesion on the cytoplasmic membrane of the organisms (Glynn and Howard, 1970). However, specific anti-capsular antibodies have been shown to activate complement leading to a bactericidal reaction (Bjornson and Michael, 1970; Goldschneider et al., 1969). It has also been shown that anti-capsular antibodies might be helpful in protecting hosts from diseases due to encapsulated organisms including Pasteurella multocida (Hofing et al., 1979) and Pasteurella haemolytica (Gilmour et al., 1983; Mukkur et al., 1983).

The purpose of these studies was to determine if development of a capsule by P. haemolytica, serotype 1 enhances resistance of the organisms to the bactericidal activity of bovine serum. The contribution of complement and antibodies in sera obtained from animals exposed naturally or experimentally to the whole organism or an extract of its capsular material were examined and discussed.

Materials and Methods

Microorganism

The organism used throughout the study was Pasteurella haemolytica, biotype A, serotype 1 originally isolated from a feedlot calf (Corstvet et al., 1973). The organisms were maintained in the lyophilized state. For each experiment, a lyophilized culture was reconstituted with distilled water and streaked for isolation onto a modified Sawata's medium (Kume et al., 1978). After 18-22 hr of incubation at 37° C in a 5% CO_2 atmosphere, isolated colonies were suspended in 0.01 M phosphate buffered saline (PBS) (pH 7.2), and the resulting suspension was streaked for confluent growth onto fresh plates of modified Sawata's

medium. These plates were incubated as described above. When growth reached the early logarithmic-phase, the cells were harvested with a cotton swab and suspended in PBS. Such suspensions typically contained encapsulated organisms (EO) as determined by Maneval's staining technique (Corstvet et al., 1982a).

Decapsulated organisms (DO) were prepared as previously described (Gentry et al., 1982). Briefly, PBS suspensions of encapsulated organisms were incubated for 1 hr on a shaking platform in a 41° C water bath. The organisms were then collected by centrifugation at 10,000 x g for 20 min and resuspended in fresh PBS. The extraction procedure reliably removed most of the capsular material from the organisms as shown by a Maneval stain of the resulting suspension. Approximate concentrations of organisms in the suspensions were determined photometrically. Actual numbers of colony forming units (CFU)/ml were determined by a spot plate method following serial dilution.

Sera

Eight bovine sera coded as AL, AC, unexposed, natural exposure A, natural exposure B, bacterin A, bacterin B, and FBS were utilized. The serum referred to as anti-live (AL) came from an animal repeatedly immunized with live P. haemolytica by both aerosol and subcutaneous injection routes. The serum referred to as anti-capsular (AC) came from an animal which was injected subcutaneously 3 times at 1 wk intervals with 18 ml of a saline capsular extract from P. haemolytica mixed with an equal volume of Freund's incomplete adjuvant. The sera designated unexposed, and natural exposure A and B came from calves which were not vaccinated, but were part of a herd in which many of

the animals had high antibody titers to P. haemolytica, apparently as a result of natural exposure to the organism. The sera designated bacterin C and D came from animals which received two 5 ml intradermal injections 1 wk apart of a formalin-killed culture of P. haemolytica (with an optical density equal to that of $1x10^9$ CFU/ml) adsorbed to aluminum hydroxide in gel adjuvant. Control sera consisted either of germ-free calf serum (kindly provided by Dr. Merwin Frey, Lincoln, NE) or commercially available fetal bovine serum (FBS) (BioLabs, Inc., Northbrook, IL). Heat-inactivated (HI) sera were prepared by incubating serum samples at 56° C for 30 min.

Measurement of serum antibody titers to somatic antigens of P. haemolytica was accomplished by a quantitative immunoassay (FIAX) as previously described (Confer et al., 1983). Results were expressed as titer values as determined by comparison to known standards. In addition, FIAX measurements of the sera were determined against a saline capsular extract from the organism prepared by a method reported previously (Gentry et al., 1982). Results were expressed as fluorescent signal units (FSU).

Samples of AL and AC sera were preadsorbed by incubation with DO for 30 min at 4° C. The organisms were then removed by centrifugation and the sera filter-sterilized.

Exogenous Complement Sources

In certain experiments lyophilized samples of guinea pig complement (Flow Laboratories, McLean, VA) were reconstituted according to manufacturer's package instructions and added to HI serum samples as a source of exogenous complement. Various dilutions of guinea pig

complement were used, such that 2.2 to 22.2 hemolytic units (as determined by the method of the National Veterinary Services Laboratories, 1979) were added to the bactericidal reaction mixtures. In other experiments, serum from a young dairy calf with a FIAX titer of 0.0 to P. haemolytica was used as a complement source. Blood was collected in a vacutainer tube and allowed to clot at 4° C for 1 hr. The serum was collected and preadsorbed with DO as above, and then immediately frozen at -70° C in small aliquots. A complement titration of the serum was performed by the method of Mueller et al. (1983) and 5.3 hemolytic complement units were added to the bactericidal reaction mixtures. HI complement sources (56° C, 30 min), which were devoid of hemolytic complement activity, were used as controls in these experiments.

Direct Bacterial Agglutination Test

The antigens used were prepared by a modification of the method of Friend et al. (1977). EO and DO were incubated at 4° C for 30 min in 0.4% formalized PBS, washed twice in PBS, and resuspended in 0.4% formalized PBS to an optical density of 1.0 at 525 nm. Two-fold serial dilutions of antisera were added to equal volumes of appropriate antigen in tubes (13 x 75 mm). All tubes were incubated at 37° C for 48 hr and examined for macroscopic agglutination.

Bactericidal Test

The reaction mixture consisted of 0.4 ml of a PBS suspension of EO or DO containing approximately $1x10^8$ colony forming units (CFU)/ml, 0.1 ml of RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand

Island, NY), and 0.1 ml of the serum to be tested. The mixture was blended vigorously on a vortex mixer for 5 sec and 0.1 ml of the mixture was immediately pipetted into 9.9 ml of PBS for a zero time sample. Additional 0.1 ml aliquots of the mixture were pipetted into 3 or 4 sterile plastic tubes that were then incubated on a shaking platform in a 37° C water bath. After various time intervals (15, 30, 45, or 60 min), a single tube was removed from the water bath and diluted with 9.9 ml of cold PBS to reduce further antibacterial action of the serum. All tubes were held on ice until viable counts could be performed by serial dilution and a spot plating technique. In certain experiments, the 0.1 ml of media was replaced by an equal volume of exogenous complement source.

The percentage change in viability at a given sampling time (n) was calculated by the following formula:

$$\% \text{ Change} = \left[\frac{\text{CFU/ml at time n} - \text{CFU/ml at time 0}}{\text{CFU/ml at time 0}} \right] \times 100$$

Thus a negative % change indicates killing of the organisms and a positive % change indicates growth of the organisms.

Statistical Analysis

Comparisons of % change in CFU/ml and FIAX reactions of sera to either formalin-killed organisms or capsular extract were made by linear regression analysis (Bailey, 1981).

Results

For the eight sera used in these studies, FIAX titers to formalin-killed organisms ranged from 0 to 652. FIAX responses to the saline capsular extract ranged from 8 to 146 FSU (Table 4.1). Agglutination

titers against both EO and DO were determined for 5 of the sera (Table 4.1). If the sera were arranged according to the descending order of their FIAX titers against formalin-killed organisms, they would also be arranged in descending order of their agglutination titers against DO. They would not be in order of their agglutination titers against EO, however, as EO was agglutinated at an 8-fold higher dilution by AC serum than by any of the other sera tested.

Curves were constructed for the % change in CFU/ml for EO and DO upon incubation with individual serum samples. Examples of typical types of curves determined for % change in CFU/ml of EO with four of the sera are shown in Figure 4.1. For 2 of the 4 sera, the number of CFU/ml decreased between 0 and 30 min, followed by apparent regrowth of surviving organisms between 30 and 60 min. Therefore, the % change in CFU/ml at 30 min was used to compare the effects of the sera tested on EO and DO. The effects of different sera on bacterial viability varied from a 98.3% reduction to a 138% increase in CFU/ml after 30 min of exposure (Figure 4.2). EO were resistant to killing by all but three of the sera: AL, AC, and Natural Exposure A. DO, however, had a decrease in CFU/ml to each of the sera tested except for FBS.

Regression analyses were performed to determine the correlation between the % change in CFU/ml after 30 min exposure and the FIAX reactions of the sera for both formalin-killed organisms (Figure 4.3) and capsular extract (Figure 4.4). DO were much more susceptible to serum killing, such that the Y intercepts for the DO regression lines were 9.9 and -2.8 respectively, as compared to 92.9 and 152.8 for EO. Also, the slopes of the regression lines are similar (-70.5 vs. -60.2,

TABLE 4.1

AGGLUTINATION AND FIAX TITERS OF BOVINE ANTISERA

Serum	Agglutination Titer Against EO	Agglutination Titer Against DO	FIAX Titer Against Formalized Organisms	FIAX Reaction (FSU) Against Capsular Extract
Anti live organisms (AL)	32	256	652	146
Anti-capsular (AC)	512	32	521	69
Fetal Bovine Serum (FBS)	--	--	0	8
Unexposed Calf	2	2	0	34
Natural Exposure A	2	8	228	64
Natural Exposure B	8	4	170	51
Bacterin C	--	--	66	33
Bacterin D	--	--	107	46

Figure 4.1. Curves of % Change in CFU/ml of Cultures of Encapsulated Organisms Exposed to Bovine Sera. Curves of % change in viability of suspensions of encapsulated organisms incubated with 16.7% bovine sera for 1 hr at 37° C. Lines represent % change in CFU/ml as determined by a viable count technique.

CURVES OF % CHANGE IN CFU/ml OF CULTURES OF ENCAPSULATED ORGANISMS EXPOSED TO BOVINE SERA

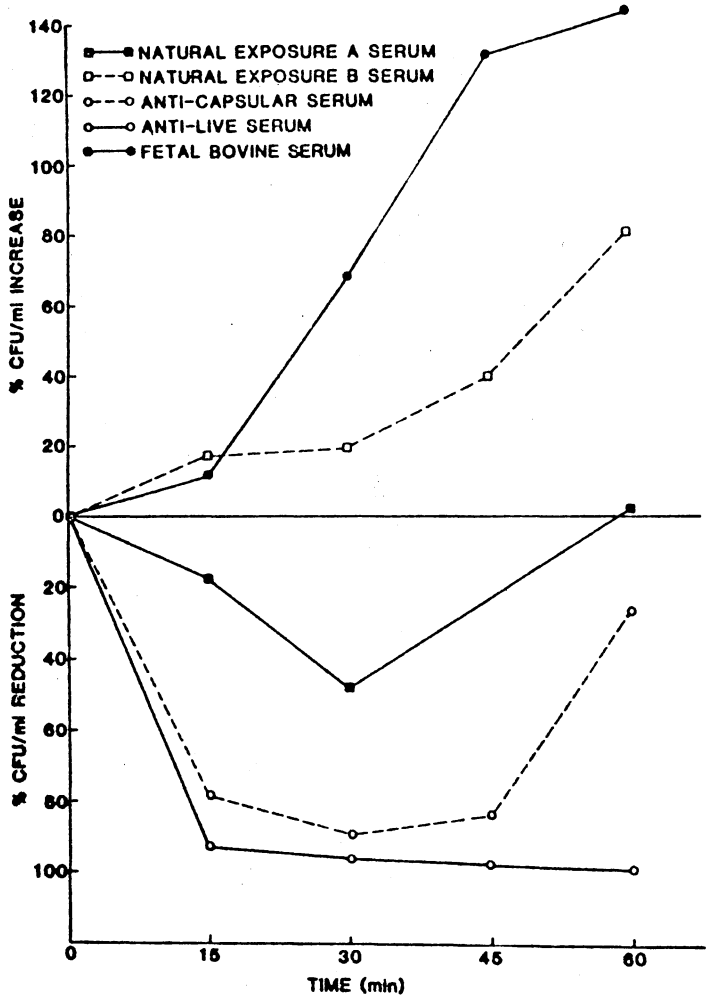


Figure 4.2. % Change in CFU/ml of Encapsulated and Decapsulated Organisms Exposed to Bovine Sera. Comparison of bactericidal action of bovine sera on encapsulated and decapsulated organisms. Bars represent % change in CFU/ml of bacterial suspensions after incubation for 30 min at 37° C in a medium containing 16.7% bovine serum.

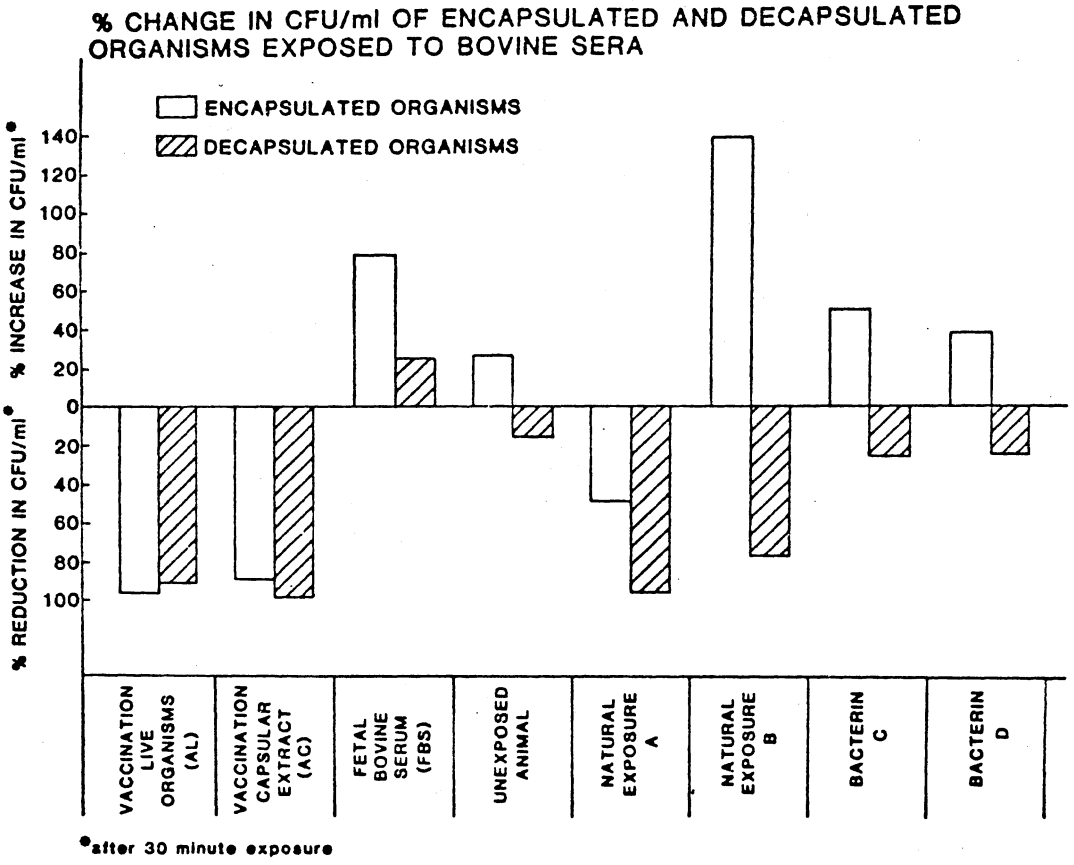

% CHANGE IN CFU/ml OF ENCAPSULATED AND DECAPSULATED ORGANISMS EXPOSED TO BOVINE SERA
ENCAPSULATED ORGANISMS
DECAPSULATED ORGANISMS
% REDUCTION IN CFU/ml* % INCREASE IN CFU/ml*
140
120
100
80
60
40
20
0
20
40
60
80
100
VACCINATION LIVE ORGANISMS (AL)
VACCINATION CAPSULAR EXTRACT (AC)
FETAL BOVINE SERUM (FBS)
UNEXPOSED ANIMAL
NATURAL EXPOSURE A
NATURAL EXPOSURE B
BACTERIN C
BACTERIN D
*after 30 minute exposure

Figure 4.3. Correlation Between % Change in CFU/ml and Log FIAX Titer to Formalin-Killed Organisms. Regression curve for % change in CFU/ml of suspensions of encapsulated or decapsulated organisms after 30 min exposure at 37° C to bovine sera vs FIAX titers of the sera to formalin-killed organisms.

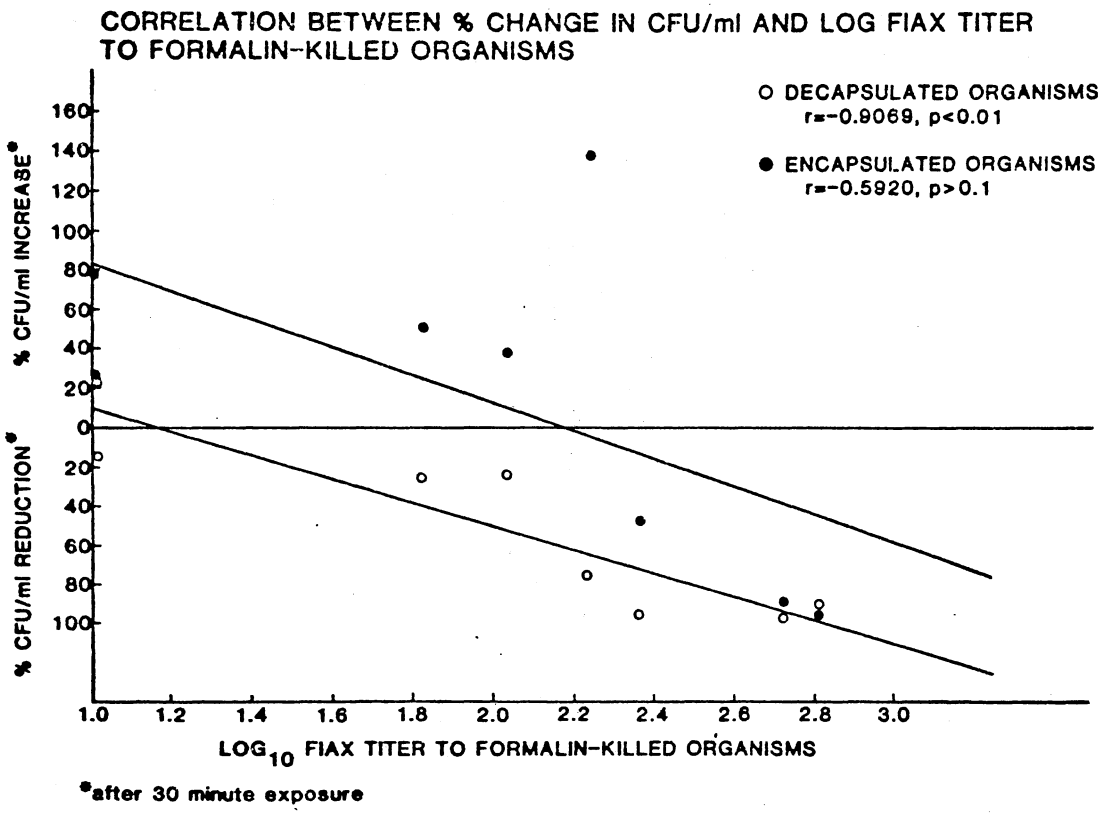
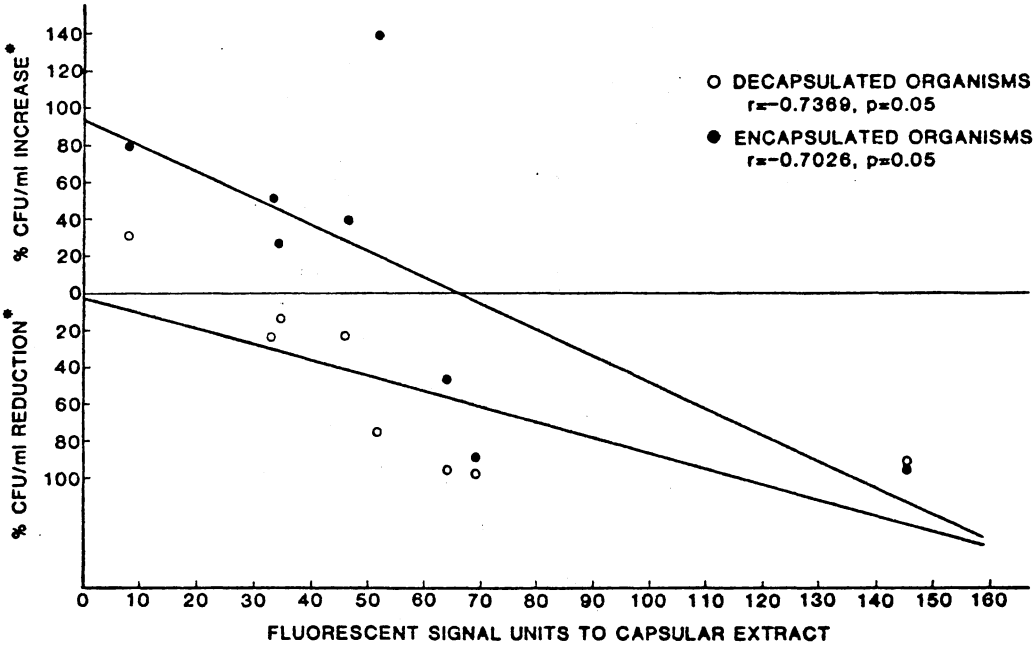

CORRELATION BETWEEN % CHANGE IN CFU/ml AND LOG FIAX TITER TO FORMALIN-KILLED ORGANISMS
O DECAPSULATED ORGANISMS
r=-0.9069, p<0.01
● ENCAPSULATED ORGANISMS
r=-0.5920, p>0.1
% CFU/ml INCREASE*
% CFU/ml REDUCTION*
160
140
120
100
80
60
40
20
0
20
40
60
80
100
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
LOG10 FIAX TITER TO FORMALIN-KILLED ORGANISMS
*after 30 minute exposure

Figure 4.4. Correlation Between % Change in CFU/ml and FIAX Reaction (FSU) to Capsular Extract. Regression curve for % change in CFU/ml of suspensions of encapsulated or decapsulated organisms after 30 min exposure at 37° C to bovine sera vs FIAX reactions (FSU) to P. haemolytica capsular extract.

CORRELATION BETWEEN % CHANGE IN CFU/ml AND
FIAX REACTION TO CAPSULAR EXTRACT



*after 30 minute exposure

and -1.43 vs. -0.84) and the lines do not intersect. The correlation coefficient (r) between the % change in CFU/ml of EO and the FIAX reaction of the sera to capsular extract ($r = -0.7026$, $p = 0.05$) was higher than that between % change in CFU/ml and the FIAX titer to formalin-killed organisms ($r = -0.5920$, $p > 0.1$). The opposite was found with DO, such that the correlation coefficient was higher between % change in CFU/ml and FIAX titer to organisms ($r = -0.9069$, $p < 0.01$) than between % change in CFU/ml and FSU to capsular extract ($r = -0.7369$, $p = 0.05$).

Heat-inactivation of the sera generally reduced, but did not prevent, their bactericidal activity. Figure 4.5 shows the effect of heating AL serum on its bactericidal activity against EO. Both the rate of killing and the total % decrease in CFU/ml at 30 min were reduced. However HI serum was still bactericidal. Addition of fresh calf serum to the reaction mixture partially restored bactericidal activity. HI serum from the same calf did not restore bactericidal activity. Similar results were found with AL serum and DO (graph not shown). Addition of fresh calf serum did not restore the original bactericidal activity to any of 5 HI serum samples tested. Results were essentially the same using guinea pig complement in place of the fresh bovine serum.

Heat-inactivation of AC serum produced a different effect than that seen for AL serum, but only when EO were utilized in the assay (Figure 4.6). Non heat-inactivated serum reduced the % CFU/ml by 89% after 30 min of exposure. Heat-inactivation completely destroyed the bactericidal capacity of AC serum, such that rapid growth of the organisms occurred. Addition of fresh calf serum or guinea pig complement partially restored the killing capability. However, addition

Figure 4.5. Effect of Heat-Inactivation of Anti-Live Serum on Encapsulated Organisms. Effect of heat-inactivation and addition of exogenous complement on the bactericidal action of anti-live serum for encapsulated organisms. Heat-inactivated serum was incubated at 56° C for 30 min.

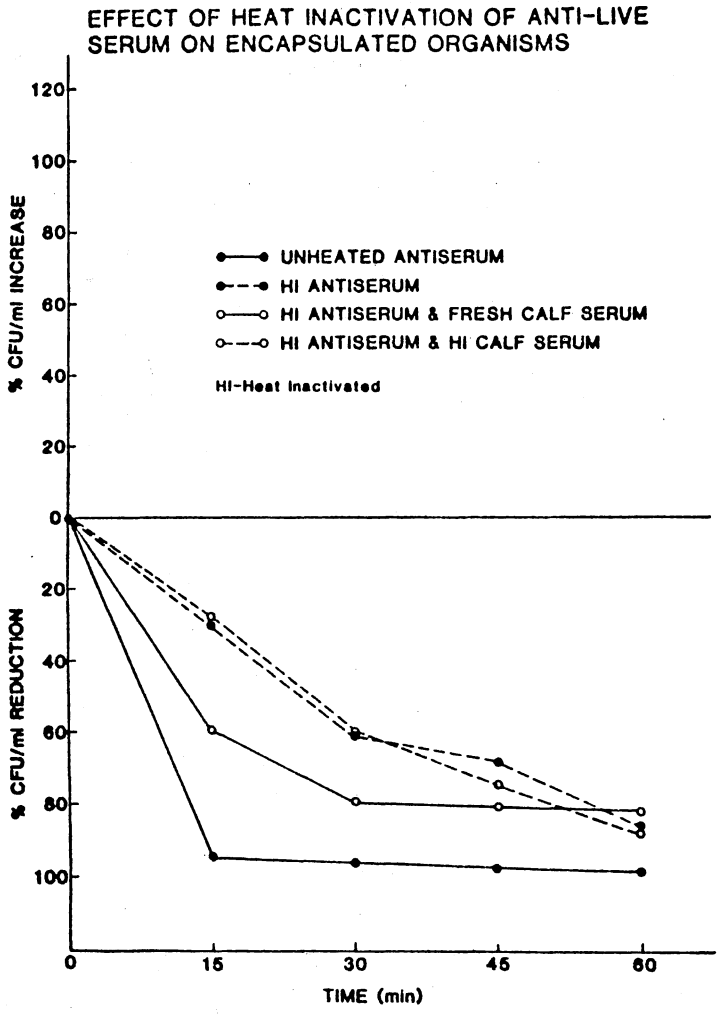
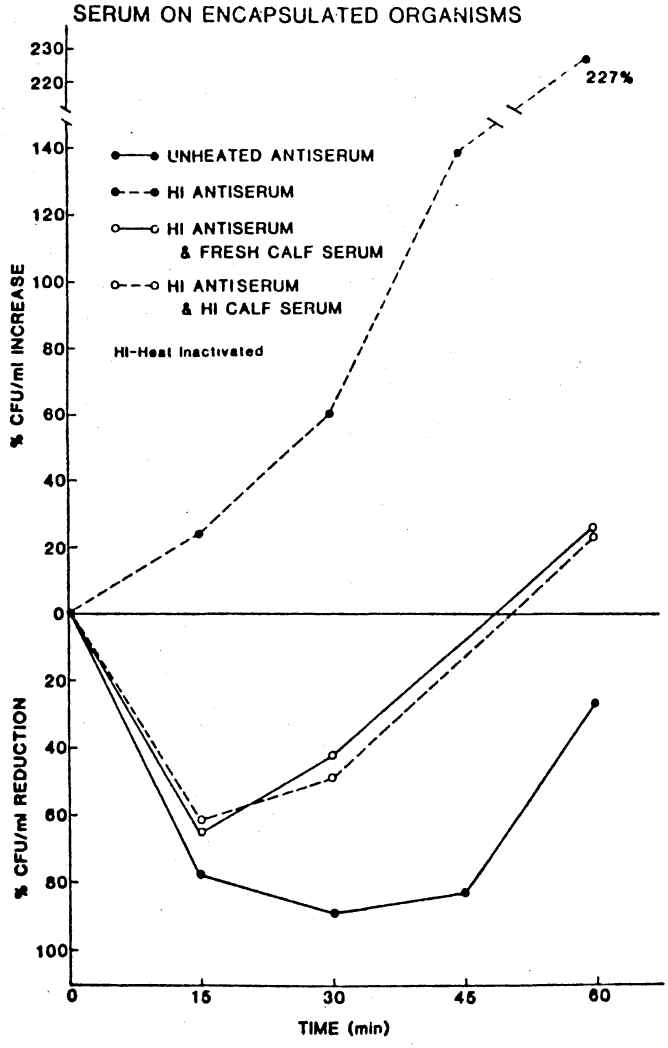

EFFECT OF HEAT INACTIVATION OF ANTI-LIVE SERUM ON ENCAPSULATED ORGANISMS
% CFU/ml INCREASE
% CFU/ml REDUCTION
120
100
80
60
40
20
0
20
40
60
80
100
UNHEATED ANTISERUM
HI ANTISERUM
HI ANTISERUM & FRESH CALF SERUM
HI ANTISERUM & HI CALF SERUM
HI-Heat Inactivated
0
15
30
45
60
TIME (min)

Figure 4.6. Effect of Heat-Inactivation of Anti-Capsular Serum on Encapsulated Organisms. Effect of heat-inactivation and addition of exogenous complement on the bactericidal action of anti-capsular serum for encapsulated organisms. Heat-inactivated serum was incubated at 56° C for 30 min,

EFFECT OF HEAT INACTIVATION OF ANTI-CAPSULAR SERUM ON ENCAPSULATED ORGANISMS



of HI calf serum or guinea pig complement also promoted killing of the organisms to the same extent. The bactericidal capability of serum from natural exposure A calf was also completely destroyed by heat-inactivation (data not shown). Addition of an exogenous complement source was not performed with that serum.

AL and AC sera, preadsorbed with DO in an attempt to remove antibodies to somatic but not capsular antigens, were compared to native sera in the direct bacterial agglutination, FIAX, and bactericidal tests with both EO and DO (Table 4.2). A substantial reduction of the direct bacterial agglutination titer occurred upon adsorption of both sera with DO. The titer of AL serum was reduced 4-fold for EO and 8-fold for DO, and the titer of AC serum was reduced 128-fold for EO and 64-fold for DO. Except for the FIAX titer of AL serum to formalin-killed organisms, the FIAX reactions of the two sera were reduced less than 2-fold. The FIAX reaction of AL serum to capsular extract was reduced by 13%. The FIAX reactions of AC serum to both formalin-killed organisms and capsular extract had reductions of 53% and 23%, respectively.

Preadsorption of AL serum caused a reduction of its bactericidal capacity for EO similar to the effect of heat-inactivation of the serum (Figure 4.7). Preadsorption effected an even greater reduction of the serum's bactericidal capacity for DO. Preadsorption of AC serum also produced a change very similar to the effect of heat-inactivation of the serum (Figure 4.8). Adsorption of that serum with DO essentially removed its bactericidal capacity for EO and substantially reduced, but did not destroy, its killing capacity for DO.

Figure 4.7. Effect of Preadsorption of Anti-Live Serum on Encapsulated and Decapsulated Organisms. Effect of preadsorption on the bactericidal action of anti-live serum for encapsulated and decapsulated organisms. Serum was preincubated for 30 min at 4° C with decapsulated organisms.

EFFECT OF PREADSORPTION OF ANTI-LIVE SERUM ON ENCAPSULATED AND DECAPSULATED ORGANISMS

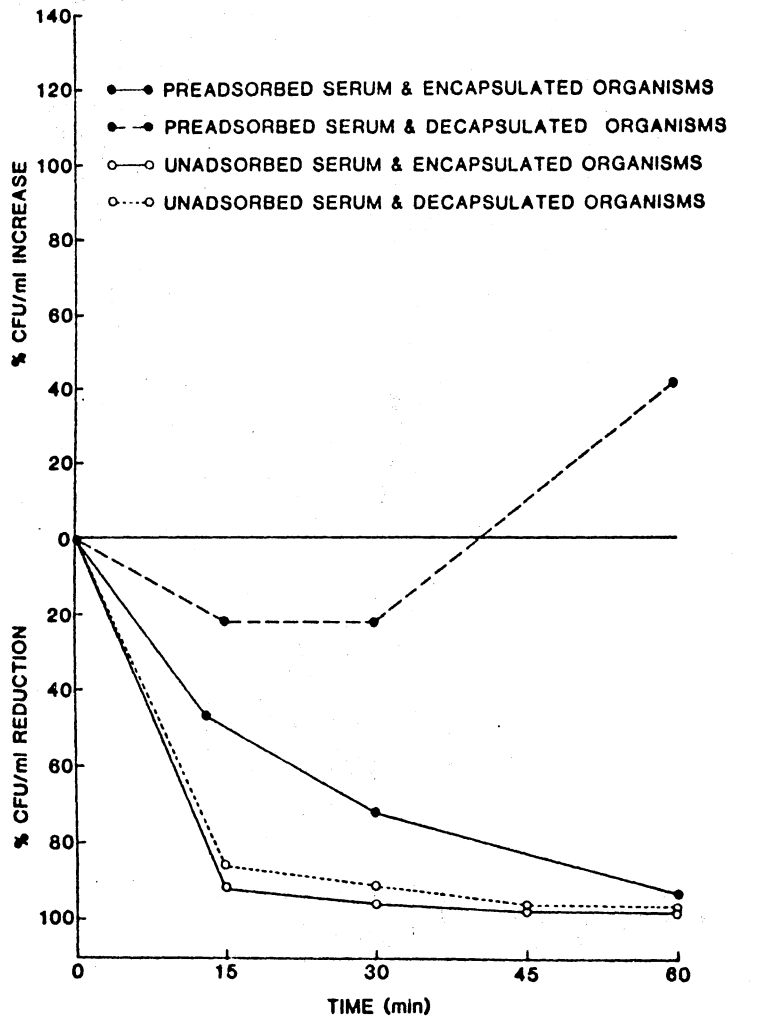


Figure 4.8. Effect of Preadsorption and Anti-Capsular Serum on Encapsulated and Decapsulated Organisms. Effect of preadsorption on the bactericidal action of anti-capsular serum for encapsulated and decapsulated organisms. Serum was preincubated for 30 min at 4° C with decapsulated organisms.

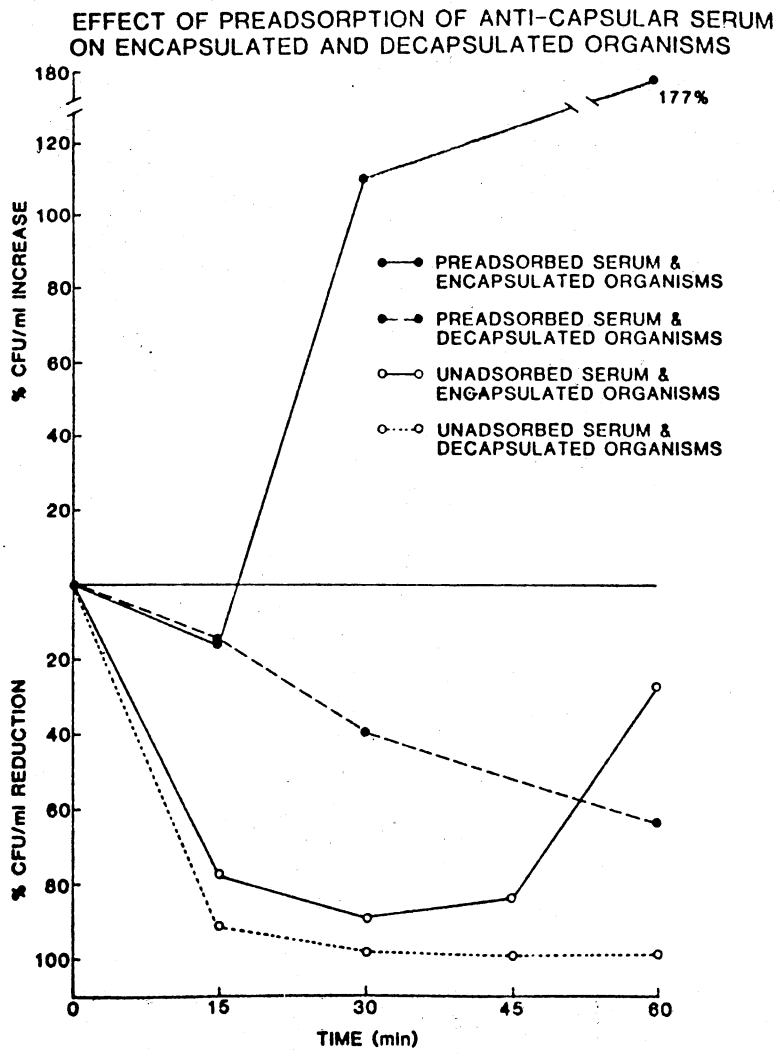

EFFECT OF PREADSORPTION OF ANTI-CAPSULAR SERUM
ON ENCAPSULATED AND DECAPSULATED ORGANISMS
% CFU/ml INCREASE
% CFU/ml REDUCTION
180
120
100
80
60
40
20
20
40
60
80
100
177%
PREADSORBED SERUM & ENCAPSULATED ORGANISMS
PREADSORBED SERUM & DECAPSULATED ORGANISMS
UNADSORBED SERUM & ENGAPSULATED ORGANISMS
UNADSORBED SERUM & DECAPSULATED ORGANISMS
0
15
30
45
60
TIME (min)

TABLE 4.2

EFFECT OF PREADSORPTION ON AGGLUTINATION AND FIAX TITERS OF ANTI-LIVE AND ANTI-CAPSULAR SERUM

Serum	Agg. Titer for EO	Agg. Titer for DO	FIAX Titer Formalized Organisms	FIAX Reaction (FSU) Against Capsular Extract
Anti-live (AL)	32	256	652	145.5
AL preadsorbed with DO	8	32	652	126.5
Anti-cap (AC)	512	128	521	69.0
AC preadsorbed with DO	4	4	247	53.0

Discussion

The two main sera utilized in the study, AL and AC, were prepared so as to have different properties. AL serum was prepared by hyper-immunizing a calf with metabolically active, encapsulated organisms. It was expected that the serum from the animal would thus contain antibodies to both somatic and capsular antigens from the organisms. AC serum was prepared by injecting a calf with a saline extract of P. haemolytica capsular material with the aim of producing a serum containing anti-capsular, but not anti-somatic antibodies. In reality, both sera possessed high FIAX titers to formalin-killed organisms. The whole organism antigen used in the FIAX test was prepared from a stationary-phase culture which showed very little capsular material upon staining (Corstvet et al., 1982a). It has also been our observation that capsular integrity is not maintained on formalin-killed organisms. The high titer demonstrated by AC serum for such an antigen indicates

that the serum indeed contains antibodies for P. haemolytica somatic antigens. Presumably, then, the capsular extract used to immunize the AC calf contained a significant quantity of somatic antigens.

AL serum reacted twice as strongly against capsular extract as did AC serum in a FIAX test. This being the case, one would expect AL serum also to have a higher agglutination titer for EO than would AC serum. To the contrary, the agglutination titer of AL serum for EO was 16-fold lower than that demonstrated by AC serum, whereas its agglutination titer for DO was 8-fold higher than the AC serum titer. Because the capsular extract used as an antigen in the FIAX test was simply a concentrate of that used for the injection of the AC calf, it is reasonable to assume that at least a portion of the FIAX response shown by AL serum for capsular extract is specific for its contaminating somatic antigens. AL serum agglutinated DO at an 8-fold higher titer than it did EO, indicating that it possess more antibodies to somatic than to capsular antigens. In contrast, AC serum agglutinated EO at a 16-fold higher titer than DO, indicating that it possesses more antibodies to capsular than to somatic antigens.

DO were found to be more sensitive than EO to the bactericidal effect of each serum tested except for AL, which was slightly more lethal for EO than DO. EO were killed only by the three sera with the highest antibody levels, whereas DO were killed by all sera except FBS. These results imply that killing of EO was due only to antibody-mediated classical complement activation, whereas killing of DO could also be accomplished by non-antibody-mediated activation of complement. Such non-antibody-mediated complement activation has been shown to contribute to the killing of rough gram negative bacteria (Taylor, 1983).

Further experiments utilizing specific blockers of the alternate and/or classical complement pathways, would be helpful in determining differences in complement activation by encapsulated versus decapsulated organisms. In previous studies, a nonencapsulated, but not an encapsulated strain of Haemophilus influenzae was also found to be sensitive to a bactericidal, non-antibody factor present in preimmune rabbit serum (Branefors and Dahlberg, 1980). Perhaps in the present studies, decapsulation of the organisms rendered them sensitive to a similar non-antibody, bactericidal factor.

The resistance of EO to the majority of sera tested appears to contradict the recent work of MacDonald et al. (1983) in which organisms in the logarithmic growth phase were found to be uniformly sensitive to the bactericidal activity of normal adult bovine blood with an indirect bacterial agglutination titer of 128. However, their reaction mixture contained 50% serum compared to 16.7% serum used in the present studies, and they found that dilution of their serum to 25% precluded the bactericidal reaction.

Serum from neither of the animals receiving bacterins was bactericidal for EO. Their FIAX reactions to capsular extract were also the lowest among the sera from animals exposed to the organisms, as might be expected from vaccination with a killed product. A comparison of the correlation coefficients between the bactericidal capacities (% change in CFU/ml) and the FIAX reactions of the sera showed that the FIAX reaction against capsular extract was a better predictor of the serum bactericidal action against EO, while the FIAX titer to formalin-killed organisms better predicted serum action against DO.

In all cases heat-inactivation of the sera that were bactericidal for DO reduced both the rate of killing and the percentage of organisms killed after 60 min of exposure. Heat treatment did not prevent their bactericidal action against DO. Heat-inactivation of 2 of the 3 sera found to be bactericidal for EO, however, rendered them completely nonbactericidal. Addition to HI antisera of complement-rich fresh calf serum that did not contain antibodies specific for P. haemolytica, in all cases restored a portion of the bactericidal action of the sera. Similar partial restoration of bactericidal activity of HI serum by exogenous complement sources has been reported for H. influenzae (Musher et al., 1983) and P. haemolytica (MacDonald at al., 1983). In the studies described herein, addition of a HI complement source, intended as a control, also enhanced the bactericidal effect of the majority of the sera tested. The reason for this phenomenon is not clear. Heat-inactivation of serum destroys only the early complement components and leaves the membrane attack complex intact. A number of workers have presented data suggesting that the membrane attack complex may be formed after activation by mechanisms distinct from either the classical or alternate pathways (Taylor, 1983). Perhaps the protease shown to be associated with the cytotoxin produced by P. haemolytica (Otulakowski, 1983) was able to enzymatically cleave the C5 complement component, leading to its activation in the absence of functional C3. As the original serum concentration used in these studies (16.7%) was lower than the 20% recommended by Taylor (1983) to ensure that killing is not limited by availability of essential complement components, the addition of extra serum (to a total serum concentration of 33.3% may simply have provided enough of the latter components to allow killing after enzymatic complement activation.

The results of the agglutination and FIAX tests for AL and AC sera which had been preadsorbed with DO indicate that adsorption was incomplete. The adsorbed sera were still capable of agglutinating both EO and DO, and also retained significant FIAX titers to formalin-killed organisms. In fact, the FIAX titer of AL serum to organisms was not reduced at all. It may be that the titer of AL serum was so high that even after reduction by adsorption, the antibody level was sufficient to exceed the upper limit of sensitivity of the FIAX system. The reduction in titer measured by the agglutination test, which is much less sensitive than the FIAX test, indicates that adsorption did reduce the antibody level of AL as well as that of AC serum.

In general, the effect of preadsorption of both AL and AC sera on their bactericidal capacity was the same as that of heat-inactivation. As mentioned above, reduction in antibody titer would not explain such an effect, because the titers were still quite high after adsorption. Perhaps adsorption removed an additional component necessary for maximum bactericidal effect. Such an adsorbable, heat-labile component was proposed earlier for the bactericidal reaction of normal human serum against nontypable Haemophilus influenzae (Musher et al., 1983). Future studies using various combinations of unadsorbed vs. adsorbed and HI vs. unheated sera would be helpful in understanding this phenomenon.

In conclusion, the capsular material on P. haemolytica from cultures in the logarithmic growth phase appears to enhance resistance of the organisms to the bactericidal effect of bovine serum. These data indicate that high antibody titers, particularly to capsular material, are needed for serum killing of encapsulated organisms to

occur, whereas decapsulated organisms are readily killed by sera even from animals with low antibody titers to the organism. It appears that complement is necessary for a maximum bactericidal effect.

CHAPTER V

INTERACTION OF ENCAPSULATED AND DECAPSULATED *PASTEURELLA HAEMOLYTICA* WITH BOVINE PHAGOCYTES

Introduction

Pasteurella haemolytica, serotype 1 is the organism which causes the acute fibrinous pneumonia associated with shipping fever or pneumonic pasteurellosis of cattle (Rehmtulla and Thompson, 1981). The organisms are commonly found in the upper respiratory tract, but not in the lungs, of healthy cattle (Magwood et al., 1969). Protection of the lung normally involves phagocytosis of organisms by resident alveolar macrophages or recruited polymorphonuclear leukocytes (PMN). However, when an animal is stressed, the organism often overwhelms the normal defense mechanisms and multiples rapidly in the lung, producing pneumonia and sometimes death (Collier, 1968). The cause of breakdown of the defense system against *P. haemolytica* is not clearly understood.

Various groups of investigators have reported wide variations in the extent of phagocytosis of *P. haemolytica* by pulmonary macrophages, which are generally considered to be the first line of defense against inhaled organisms. One group using logarithmic-phase cultures of *P. haemolytica* was unable to demonstrate uptake of ^{14}C-labelled organisms (Walker et al., 1980). Another group using formalin-killed 18-hr cultures reported that *P. haemolytica* was poorly phagocytized by

cultured bovine alveolar macrophages (Markham and Wilkie, 1980). The addition of immune serum enhanced uptake somewhat. Those authors suggested that when large numbers of organisms are presented to the lung following stress-related depression in macrophage function, the bacteria themselves may further impair macrophage viability and phagocytosis. Others (Maheswaran et al., 1980) have reported high levels of phagocytosis of P. haemolytica by bovine alveolar macrophages adhered to plastic. Up to 15% of stationary-phase organisms were reportedly phagocytized without prior opsonization, and 90-95% of organisms were phagocytized after opsonization with normal or immune serum, respectively. Perhaps the difference between results from these groups derives from the age of the cultures utilized. Berggren et al. (1981) compared the interaction of bovine PMN with logarithmic- and stationary-phase cultures of P. haemolytica opsonized with normal bovine serum or antiserum. They found that only 5-8% of logarithmic-phase organisms were phagocytized, whereas 90-98% of stationary-phase organisms were phagocytized at bacteria:PMN ratios of 10:1.

Many important pathogenic bacteria possess capsular or cell wall components which inhibit phagocytosis (Karakawa and Young, 1979; Densen and Mandell, 1980; Hill et al., 1983; William et al., 1983). In many such organisms, resistance to phagocytosis is considered to be their main virulence mechanism (Smith, 1977). Capsular material has been shown to prevent efficient phagocytosis of Pasteurella multocida by bovine PMN unless hyperimmune serum was used as an opsonic source (Maheswaran and Thies, 1979). Enzymatic decapsulation of the organisms, however, rendered them susceptible to phagocytosis. Physical reduction of the amount of capsular material on one strain of Klebsiella pneumoniae

was also found to increase its susceptibility to opsonization and subsequent phagocytosis (Williams et al., 1983).

P. haemolytica from logarithmic-phase cultures have been reported to be encapsulated, whereas stationary-phase cultures were composed mainly of nonencapsulated organisms (Corstvet et al., 1982a). The difference in capsule content of organisms from cultures at different stages of the growth cycle may have a bearing upon their reported difference in resistance to phagocytosis. The purpose of the present study was to compare the interactions of encapsulated and decapsulated P. haemolytica with bovine phagocytes. Differences in opsonization by immune and nonimmune sera, extent of phagocytosis, fate of internalized organisms and cytotoxicity of organisms for phagocytes were examined. The findings are discussed in terms of a possible role for capsular material in resistance of P. haemolytica to phagocytosis by bovine phagocytes.

Materials and Methods

Microorganism

The organism used throughout the study was P. haemolytica, biotype A, serotype 1 originally isolated from a feedlot calf (Corstvet et al., 1973). The bacteria were maintained in the lyophilized state. The organisms were grown on a modified Sawata's medium (Kume et al., 1978), and suspensions of both encapsulated organisms (EO) and decapsulated organisms (DO) were prepared in phosphate buffered saline (PBS) by the methods outlined in Chapter IV.

Sera

The six bovine sera used in the serum bactericidal assays were used in these studies also (Chapter IV). The sera are designated as follows: anti-live serum (AL), anti-capsular serum (AC), fetal bovine serum (FBS), unexposed serum, and natural exposure A and B serum. Measurement of direct bacterial agglutination titers, and serum antibody titers to somatic antigens or to a capsular extract of P. haemolytica were performed as described in Chapter IV. All sera utilized in these studies had been heat-inactivated by incubation at 56° C for 30 min.

Samples of AL and AC sera were preadsorbed by incubation with formalin-killed DO as in Chapter IV. Crude IgG fractions of AL and AC sera were also prepared by $(NH_4)_2SO_4$ precipitation as described in Chapter VI.

Chicken antiserum was prepared against P. haemolytica cytotoxin-B as described previously (Gentry et al., 1982).

Bovine Phagocyte Preparation

Polymorphonuclear leukocytes (PMN) were collected from heparinized blood from healthy cattle on Ficoll-Paque (Pharmacia, Piscataway, NJ) as described in Chapter VI. Pulmonary alveolar macrophages (PAM) were collected from healthy cattle by bronchoalveolar lavage as described previously (Corstvet et al., 1982b). Suspensions of the phagocytes in RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, NY) were prepared as related in Chapter VI.

Opsonization of Organisms and Phagocytosis Assay

Opsonization and phagocytosis assays were performed by a

modification of the method of Rush et al. (1981) in sterile 5 ml siliconized glass tubes. Opsonization mixtures consisted of 0.04 ml of appropriate bacterial suspension containing 5 x 10^7 to 2 x 10^8 colony-forming-units (CFU)/ml, 0.1 ml of RPMI 1640, and 0.1 ml of bovine serum. The mixtures were incubated for 15 min on a shaking platform in a 37° C water bath. To each tube, 0.4 ml of a suspension of bovine phagocytes was then added at organism to cell ratios ranging from 4:1 to 45:1. The tube contents were vigorously mixed for 5 sec on a vortex mixer (Scientific Products, Inc., Bohemia, NY) and 0.1 ml of the mixture was immediately withdrawn and added to 9.9 ml of PBS at 4° C for a zero time (T_0) viable count sample. All tubes were held on ice until the phagocytes could be added to each one. When all additions had been completed, the tubes were incubated again for 15 min at 37° C as described previously to allow phagocytosis to occur. The tubes were then centrifuged at 4° C for 10 min at 115 x g to separate the phagocytes from unphagocytized organisms (differential centrifugation). The supernatants were carefully removed with a Pasteur pipette and discarded. The cell pellets were washed three times in cold PBS (4° C) with differential centrifugation between washes. Supernatants from the washes were aspirated and discarded. The final phagocyte pellets were resuspended in RPMI 1640 to their original volume (0.9 ml). From each tube, a 0.1 ml sample was removed and added to a separate 9.9 ml volume of cold distilled water (4° C) containing 0.01% gelatin (Eastman Kodac Co., Rochester, NY). The gelatin-water samples were then vigorously mixed for 15 sec on a vortex mixer to lyse the phagocytes, and held on ice for viable counts designated "Lyl." All samples on which counts were

performed were held on ice until viable counts could be performed by a spot-plate technique after serial dilutions of the samples in PBS.

Calculation of % phagocytosis was determined by the following formula:

$$\% \text{ Phagocytosis} = \left(\frac{\text{CFU/ml in Lyl sample}}{\text{CFU/ml in } T_0 \text{ sample}} \right) \times 100$$

An experiment was performed to verify the validity of the differential centrifugation and viable counting techniques as a measure of % phagocytosis. The method followed was that of van Furth and van Zwet (1978). The experimental design was essentially the same as described previously, except that six different viable count measurements were performed as follows:

Sample A--Same as T_0 count above, 0.1 ml of mixture of organisms and phagocytes before incubation added to 9.9 ml of PBS to determine initial number of CFU/ml.

Sample B--0.1 ml of mixture of organisms and phagocytes after incubation added to 9.9 ml of distilled water to determine total number of CFU/ml.

Sample C--0.1 ml of same mixture as in Sample B added to 9.9 ml of PBS to determine number of CFU/ml of extracellular organisms only.

Sample D--0.1 ml of resuspended phagocyte pellets after final differential centrifugation wash added to 9.9 ml of PBS to determine number of pellet-associated extracellular CFU/ml.

Sample E--0.1 ml of same suspension as in Sample D added to 9.9 ml of distilled water to determine total number of pellet-associated CFU/ml.

In these experiments, results were compared of % phagocytosis as calculated by three different methods as follows:

$$\text{Method 1:}^{*}\ \%\ \text{phagocytosis} = \left(\frac{\text{CFU/ml in Sample E}}{\text{CFU/ml in Sample A}}\right) \times 100$$

$$\text{Method 2:}\ \%\ \text{phagocytosis} = \left(\frac{\text{CFU/ml in Sample E}}{\text{CFU/ml in Sample B}}\right) \times 100$$

$$\text{Method 3:}\ \%\ \text{phagocytosis} = \left(\frac{\text{CFU/ml in Sample E} - \text{CFU/ml in Sample D}}{\text{CFU/ml in Sample B}}\right) \times 100$$

*Method used in original experiments

Additional calculations were performed as follows:

$$\text{Measurement of Agglutinated Organisms in Cell Pellet} = \text{CFU/ml in Sample D}$$

$$\text{Measurement of Intracellular Organisms After Phagocytosis Incubation} = \text{CFU/ml in Sample B} - \text{CFU/ml in Sample C}$$

$$\text{Measurement of Intracellular Organisms After Differential Centrifugation} = \text{CFU/ml in Sample E} - \text{CFU/ml in Sample D}$$

Fate of Phagocyte-Associated Organisms

Experiments were performed using resuspended phagocytic cells after the final wash step of the opsonization and phagocytosis assays. After removal of the Ly1 viable count samples, the tubes of suspended phagocytes were incubated for 60 additional min on a shaking platform in a 37° C water bath. From each tube, a 0.1 ml sample was removed after 30 min and again after 60 min, and added to a separate 9.9 ml volume of ice-cold 0.01% gelatin-water. The gelatin-water samples were vigorously mixed for 15 sec on a vortex mixer to lyse the

phagocytes and held on ice for viable count measurements designated "Ly2" and "Ly3" respectively. Viable count measurements were performed as described previously. Calculations of change in viability of cell-associated organisms were determined by the following formulas:

$$\text{Change in viability of cell-associated organisms after 30 min} = \left(\frac{\text{CFU/ml in Ly2 Sample} - \text{CFU/ml in Ly1 Sample}}{\text{CFU/ml in Ly1 Sample}} \right) \times 100$$

$$\text{Change in viability of cell-associated organisms after 60 min} = \left(\frac{\text{CFU/ml in Ly3 Sample} - \text{CFU/ml in Ly1 Sample}}{\text{CFU/ml in Ly1 Sample}} \right) \times 100$$

In several experiments, attempts were made to prevent growth of any extracellular cell-associated organisms; 25-100 units/ml of Penicillin plus 25-100 µg/ml of Streptomycin (Pen-Strep, GIBCO Laboratories, Grand Island, NY) were added to the RPMI 1640 medium that was used to resuspend the phagocyte pellets after the final wash by differential centrifugation.

Toxicity of Organism for Phagocytes

Determination of viability of phagocytes was performed concurrently with the measurements of fate of cell-associated organisms using the same phagocyte suspensions. Thus at the time of removal of the Ly1, Ly2, and Ly3 samples (0, 30, and 60 min), an additional 0.1 ml sample of each phagocyte suspension was removed and added to a separate 0.1 volume of cold RPMI 1640. The tubes were held on ice for trypan blue exclusion determinations of phagocyte viability. Immediately before each sample was counted, 0.1 ml of 0.4% trypan blue (GIBCO Laboratories,

Grand Island, NY) was added, and 200-300 cells were examined microscopically for viability using a hemacytometer.

Once again, in certain experiments various concentrations of Pen-Strep were added to the RPMI medium in an attempt to prevent growth of extracellular organisms (and thus preclude cytotoxin formation. In still other experiments, the RPMI 1640 used to resuspend the phagocytes was supplemented with 33% chicken anti-cytotoxin B serum as a means of protecting the phagocytes from cytotoxin produced *in situ*.

Preparation of Samples for Transmission Electron Microscopy

Samples of pulmonary alveolar macrophages (PAM) in RPMI 1640 processed through the final wash step of the opsonization and phagocytosis assay using encapsulated organisms and AL serum, were centrifuged at 15,000 rpm in a microcentrifuge (Epperdorf Co., Hamberg, West Germany) and resuspended in cacodylate-buffered 1% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) for 1 hr. The cells were then washed in three 15 min changes of cacodylate buffer, post-fixed for an additional 1 hr in 2% osmium tetroxide (Stevens Metallurgical Corp., New York, NY), and dehydrated in a graded ethanol series. The dehydrated cells were embedded in Polybed (Polysciences Inc., Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Phillips 200 transmission electron microscope.

Statistical Analysis

Comparisons of % phagocytosis to agglutination titers or to FIAX

titers of the sera used for opsonization were performed by linear regression analysis, as were comparisons of % decrease in phagocyte viability to % phagocytosis or to % increase in CFU/ml of phagocyte suspensions (Bailey, 1981). Cytotoxicity of EO and DO for PAM were compared by Student's t-test.

Results

Opsonization and Phagocytosis Assays

When phagocytic capacity of PMN and PAM were compared in experiments with the same kind of organisms opsonized with the same antiserum, the % phagocytosis was consistently 1.1-2.5 times higher when PAM were used than when PMN were used (Table 5.1).

TABLE 5.1

COMPARISON OF PHAGOCYTOSIS BY BOVINE PMN AND PAM

Organism Used	Serum Used	Avg. % Phagocytosis In PMN Experiments	Avg. % Phagocytosis In PAM Experiments	Avg. % with PAM / Avg. % with PMN
Encapsulated	FBS	0.4 (4)	1.0 (4)	2.5
Decapsulated	FBS	0.8 (4)	1.8 (4)	2.2
Encapsulated	AL	23.1 (4)	49.9 (16)	2.2
Decapsulated	AL	6.6 (4)	14.0 (6)	2.1
Encapsulated	AC	143.0 (4)	160.3 (10)	1.1
Decapsulated	AC	9.0 (4)	16.1 (7)	1.8

Note: ( ) indicates number of trials averaged.

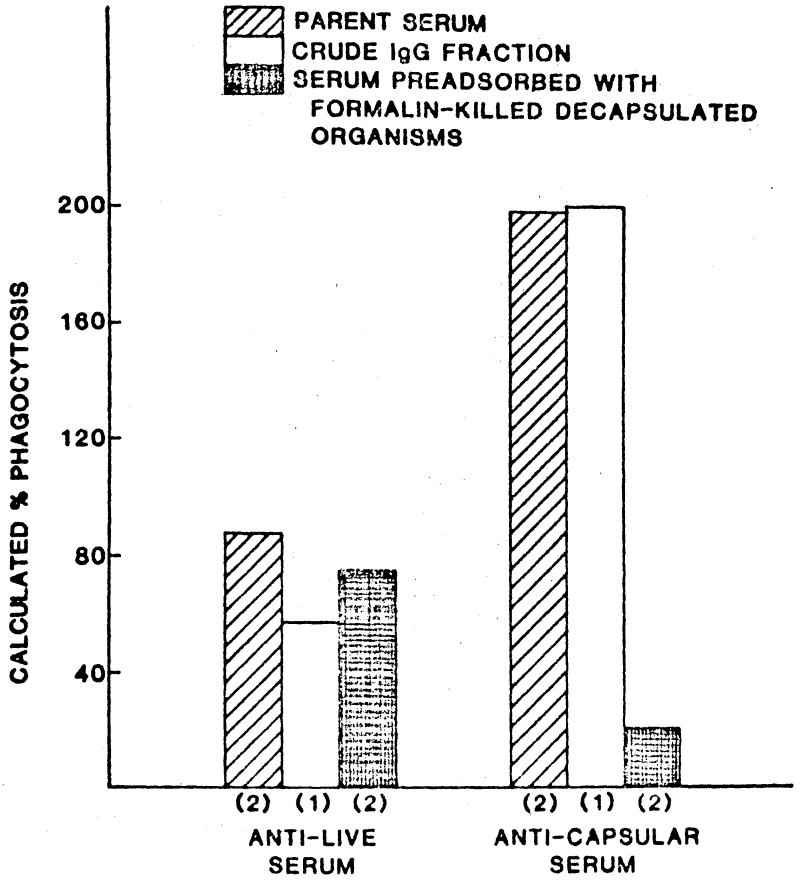
FBS was a poor opsonin for either EO or DO, allowing for less than 2% phagocytosis by PMN or PAM, whereas opsonization with AL or AC serum increased phagocytosis of both EO and DO (Table 5.1). In experiments where EO and DO were compared after opsonization by AL serum, the % phagocytosis of EO was 2.0 and 3.5 times higher than that calculated for DO, using PAM and PMN respectively. Using AC serum, the average % phagocytosis calculated for EO was 9.8 and 10.0 times higher than that calculated for DO, using PAM and PMN respectively (data used to calculate % phagocytosis for EO were taken only from experiments in which both cell types were used). Overall, when AL and AC sera were used with either type of phagocyte, DO appeared to be opsonized equally well by both sera, whereas EO appeared to be opsonized 3.2-4.2 times better by AC serum than by AL serum.

Phagocytosis of EO by PAM was studied following opsonization with IgG fractions and preadsorbed sera obtained from AL and AC sera (Figure 5.1). The IgG fraction from AC serum opsonized EO as efficiently as the parent serum, whereas phagocytosis of organisms opsonized with the IgG fraction of AL serum was approximately 64% of that calculated when the native serum was used. Preadsorption of AL serum with formalin-killed DO only slightly reduced its apparent opsonizing capacity for EO, whereas preadsorption of AC serum with formalin-killed DO reduced the calculated % phagocytosis by approximately 89% (Table 5.2).

The % phagocytoses of EO by PAM after opsonization with unexposed, natural exposure A and natural exposure B sera are found in Table 5.2. The % phagocytosis after opsonization with unexposed serum was lower than that after opsonization with either natural exposure serum.

Figure 5.1. Calculated % Phagocytosis with Macrophages Using Treated Sera to Opsonize Encapsulated Organisms. Comparison of % phagocytosis by bovine macrophages of encapsulated organisms opsonized by anti-live or anti-capsular native serum, preadsorbed serum, or serum IgG fraction. Preadsorbed sera were preincubated for 30 min at 4° C with decapsulated organisms. Serum IgG fractions were isolated by $(NH_4)_2SO_4$ precipitation.

CALCULATED % PHAGOCYTOSIS WITH MACROPHAGES USING TREATED SERA TO OPSONIZE ENCAPSULATED ORGANISMS



( ) indicates number of trials

TABLE 5.2

COMPARISON OF IMMUNOASSAYS FOR CRUDE IgG FRACTIONS AND PREADSORBED BOVINE SERA VERSUS THEIR NATIVE SERA

Serum	Calculated % Phagocytosis	Agg. Titer for EO	FIAX Titer Against Whole Organisms	FIAX Reaction Against Capsular Extract
AL	87.5	32	652	145.5
AL-Ad*	75.4	8	652	126.5
AC	198.0	512	521	69.0
AC-Ad*	21.7	4	247	53.0
Unexposed	5.4	2	0	34.5
Natural Exposure A	27.4	2	228	64.0
Natural Exposure B	59.0	8	170	51.5

*Sera preadsorbed with formalin-killed decapsulated organisms.

Regression analyses were performed between % phagocytosis and three other immunological measurements of antibody to P. haemolytica (Table 5.2). % phagocytosis correlated positively ($r = 0.9794$, $p < 0.001$) with the direct bacterial agglutination titer of the sera, but did not correlate with the FIAX titer to formalin-killed P. haemolytica ($r = 0.6887$, $p > 0.05$) or to the FIAX reaction to P. haemolytica capsular extract ($r = 0.3211$, $p > 0.2$).

To determine if agglutinated organisms were being pelleted with phagocytes during differential centrifugation, an experiment was performed using PAM and either EO or DO opsonized with FBS, AL serum or AC serum. The % phagocytosis was calculated by three different methods (Table 5.3). A comparison of methods 1 and 2 was made to determine the contribution of increase in viable count of the organism

suspension during opsonization to the calculated % phagocytosis. The % phagocytoses calculated by the two methods were comparable for all samples except EO opsonized with AC serum. A comparison of method 3 to method 2 was made to determine the contribution of agglutinated organisms to the calculated % phagocytosis. Calculation by method 3 could not be performed using DO and AL serum. For four of the other five samples, the % phagocytosis calculated by method 2 and method 3 were comparable. In the sample using DO and AC serum, the % phagocytosis calculated by method 3 was considerably less than that calculated by method 2.

TABLE 5.3

COMPARISON OF % PHAGOCYTOSIS CALCULATED BY THREE DIFFERENT METHODS

Organisms	Serum	% Phagocytosis Calculated By		
		Method 1*	Method 2	Method 3
Encapsulated	FBS	0.9	0.4	0.03
Decapsulated	FBS	1.4	1.6	0.65
Encapsulated	AL	38.2	45.2	41.9
Decapsulated	AL	8.8	8.4	NC¶
Encapsulated	AC	111.1	50.0	48.0
Decapsulated	AC	16.4	20.3	9.2

*Method used in original studies.

¶Not calculated--viable count D was higher than viable count E.

The number of viable extracellular, PAM-associated organisms was found to be approximately the same for all samples opsonized with AL or AC sera (Table 5.4). The number of such organisms in the samples

opsonized with FBS was 0.3 to 1.0 log units less than any of the samples opsonized with antiserum. The number of viable intracellular organisms as determined by CFU/ml after the phagocytosis incubation, was approximately 1.0-1.5 log units higher for EO opsonized with antisera than for any of the other samples. The number of viable intracellular organisms after the final wash of the differential centrifugation was approximately 0.2-1.0 log units higher for EO opsonized with antisera than for any of the other samples.

TABLE 5.4

COMPARISON OF EXTRACELLULAR AND INTRACELLULAR PAM-ASSOCIATED ORGANISMS

Organisms	Serum	CFU/ml of Extracellular PAM-Associated Organisms	CFU/ml of Intracellular Organisms I*	CFU/ml of Intracellular Organisms I**
Encapsulated	FBS	1.2×10^5	1.0×10^4	2.0×10^6
Decapsulated	FBS	4.0×10^4	2.8×10^4	4.0×10^5
Encapsulated	AL	3.0×10^5	3.9×10^6	4.0×10^6
Decapsulated	AL	5.5×10^5	NC***	1.3×10^6
Encapsulated	AC	4.1×10^5	9.6×10^6	1.7×10^7
Decapsulated	AC	4.2×10^5	3.5×10^5	1.1×10^6

*Calculated by measurements taken at end of phagocytosis incubation.

**Calculated by measurements taken after final wash of differential centrifugation.

***NC = Not calculated--viable count D was higher than viable count E.

A comparison of the number of extracellular phagocyte-associated and viable intracellular organisms after differential centrifugation revealed that in the samples containing EO opsonized with either AL or AC serum, the number of intracellular organisms calculated was 1.0-1.5 log units higher than the number of extracellular organisms (Table 5.4). In all of the other samples, the number of CFU/ml was found to be slightly higher for extracellular than for intracellular organisms. A comparison of the number of calculated intracellular organisms in the samples after the phagocytosis incubation step with the number calculated after the final wash by differential centrifugation, revealed a decrease for all samples of from 0.1 to 2.5 log units.

Fate of Phagocyte-Associated Organisms

The viable count of phagocyte-associated organisms in phagocyte suspensions after differential centrifugation increased after an additional incubation period, regardless of what combination of phagocyte, organism, and antiserum was used (Table 5.5). There was no apparent consistent difference in extent of growth between EO and DO. Addition of 25 units/ml of Penicillin and 25 µg/ml of Streptomycin to the incubation medium reduced the extent of organism proliferation in all samples. An increase in antibiotic concentration to 100 units/ml Penicillin and 100 µg/ml Streptomycin provided essentially the same result.

In samples using EO plus AC serum or DO plus AL serum, a viable count decrease occurred when antibiotics were added to the medium. In the other samples, viable count increases occurred (Table 5.5).

TABLE 5.5

CHANGE IN CFU/ml OF SUSPENSIONS OF PHAGOCYTES
DURING POST-PHAGOCYTOSIS INCUBATION

Phagocytes	Organisms	Serum	% Change in CFU/ml After 60 min Incubation Without Antibiotics	% Change in CFU/ml After 60 min Incubation With Antibiotics
PMN	Encapsulated	FBS	+164 (3)	NT
PMN	Decapsulated	FBS	+159 (3)	NT
PMN	Encapsulated	AL	+199 (2)	+169 (1)
PMN	Decapsulated	AL	+68 (2)	-72 (1)
PMN	Encapsulated	AC	+109 (2)	-15 (1)
PMN	Decapsulated	AC	+572 (2)	+75 (1)
PAM	Encapsulated	FBS	+156 (4)	NT
PAM	Decapsulated	FBS	+186 (4)	NT
PAM	Encapsulated	AL	+886 (9)	+285 (4)
PAM	Decapsulated	AL	+244 (4)	-27 (2)
PAM	Encapsulated	AC	+154 (6)	-37 (3)
PAM	Decapsulated	AC	+859 (4)	+52 (3)

Note: NT = Not tested; ( ) indicates number of trials averaged.

Transmission electron microscopy was performed on PAM suspensions after phagocytosis of EO opsonized with AL serum. Extracellular organisms were seen as well as organisms in various stages of degeneration within phagocytic vacuoles (Figures 5.2 and 5.3). Both viable and degenerated PAM were present.

Cytotoxicity of Encapsulated and Decapsulated Organisms for Phagocytes

Phagocyte viability decreased during a 60 min post-phagocytosis incubation of all samples (Table 5.6). EO and DO were equally toxic

Figure 5.2. Electron Micrograph of Pulmonary Alveolar Macrophage Phagocytizing Encapsulated Organisms. Undegraded organisms may be seen within membrane-bound phagocytic vacuoles (x 31,250).

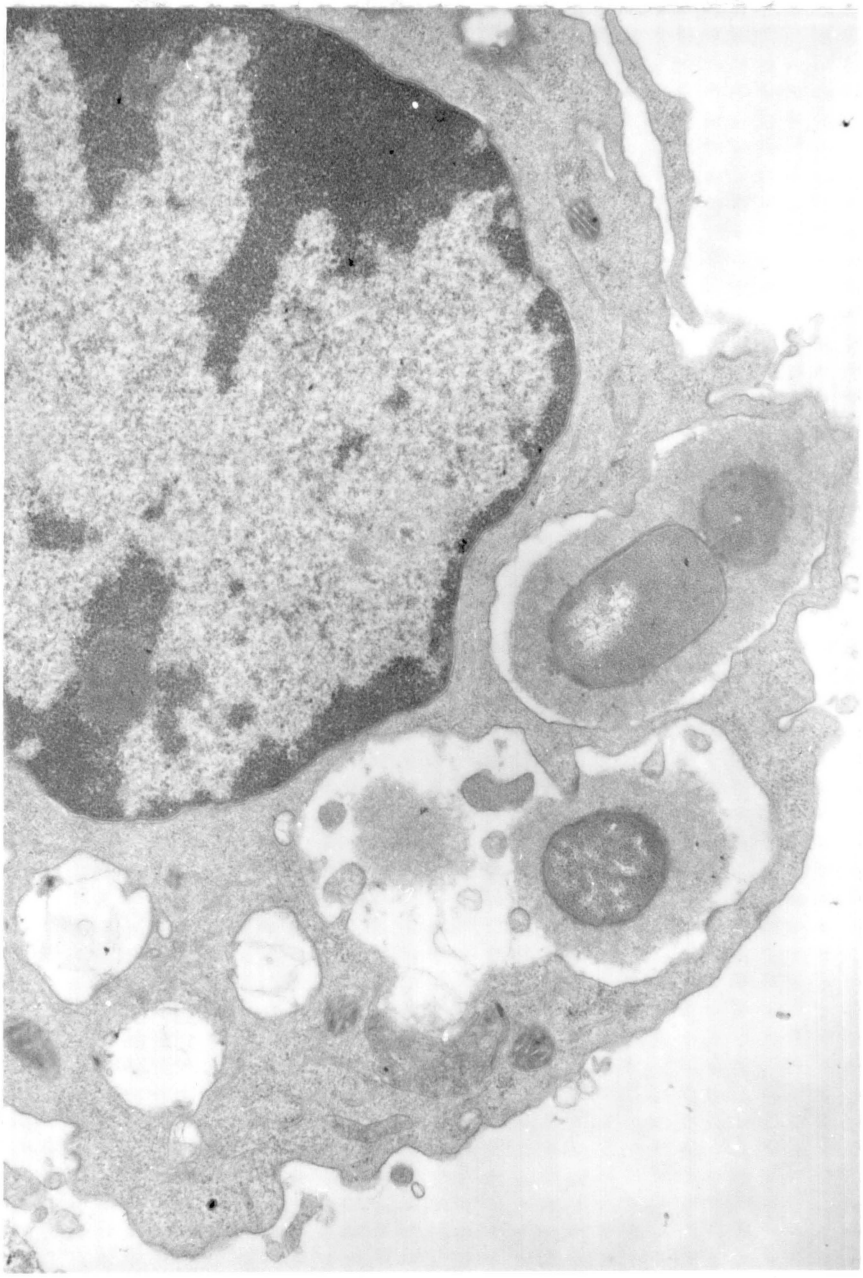


Figure 5.3. Electron Micrograph of Pulmonary Alveolar Macrophage Degrading Phagocytized Encapsulated Organisms. Electron micrograph of bovine macrophage after phagocytosis of encapsulated organisms. Organisms are in various states of degradation within phagocytic vacuoles. Intact extracellular encapsulated organisms are also present (x 24,750).

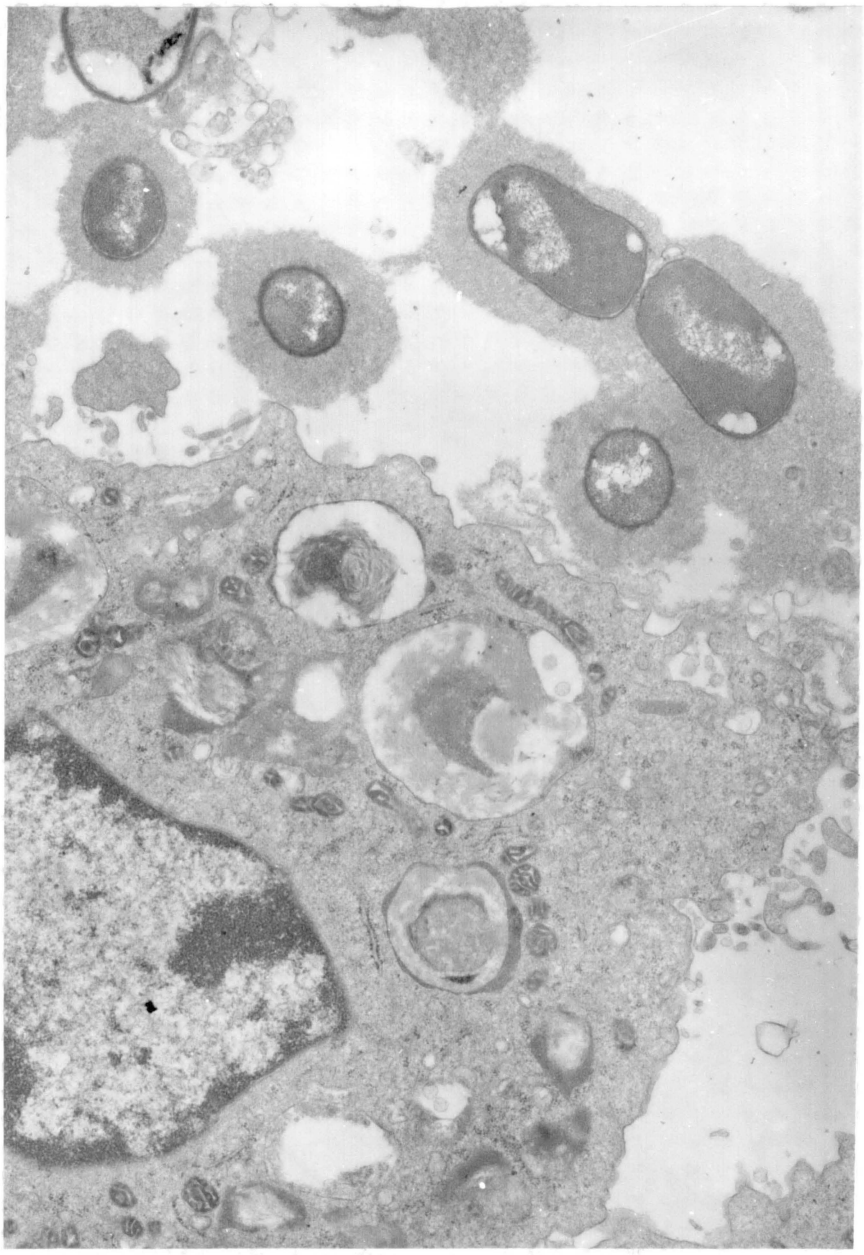


TABLE 5.6

DECREASE IN PHAGOCYTE VIABILITY DURING POST-PHAGOCYTOSIS INCUBATION

Phagocytes	Organisms	Serum	% Phagocytes Viable Before Incubation	% Phagocytes Viable After 1-hr Incubation	% Decrease in Viability Without Antibiotics	% Decrease in Viability With Antibiotics Added
PMN	Encapsulated	FBS	0.0 (2)	No cells seen (2)	--	NT
PMN	Decapsulated	FBS	0.0 (2)	No cells seen (2)	--	NT
PMN	Encapsulated	AL	84.9 (3)	24.0 (2)	60.9 (2)	59.7 (1)
PMN	Decapsulated	AL	85.2 (3)	19.9 (2)	65.3 (2)	58.5 (1)
PMN	Encapsulated	AC	73.2 (3)	23.7 (2)	49.5 (2)	48.6 (1)
PMN	Decapsulated	AC	78.1 (3)	24.8 (2)	53.2 (2)	42.8 (1)
PAM	Encapsulated	FBS	49.5 (4)	32.1 (4)	16.9 (4)	NT
PAM	Decapsulated	FBS	69.8 (4)	54.8 (4)	15.0 (4)	NT
PAM	Encapsulated	AL	60.2 (10)	25.6 (10)	34.6 (10)	27.9 (4)
PAM	Decapsulated	AL	67.3 (4)	54.4 (4)	12.9 (4)	11.2 (2)
PAM	Encapsulated	AC	70.2 (7)	29.9 (7)	40.3 (7)	39.0 (3)
PAM	Decapsulated	AC	70.7 (4)	42.7 (4)	28.0 (4)	14.5 (3)

Note: NT = Not tested; ( ) indicates number of trials averaged.

to PMN using all sera tested. EO were more toxic to PAM than were DO after opsonization by AL or AC serum, but not after opsonization with FBS. The difference was statistically significant ($p = 0.04$) in samples using AL serum, but was not significant ($p = 0.06$) in samples using AC serum. In parallel samples both EO and DO were more toxic for PMN than for PAM. Live PMN could not be found in samples using either EO or DO opsonized with FBS. Addition of Pen-Strep to the incubation medium slightly reduced the % decrease in viability for all samples tested (Table 5.6). In no case did Pen-Strep completely prevent phagocyte death. In an experiment in which PAM were incubated with EO opsonized with AL serum, supplementation of the incubation medium with 33% chicken anti-cytotoxin-B serum reduced the % decrease in viability by 6.5% but did not prevent macrophage death (data not shown).

Discussion

Phagocytosis studies were carried out using both bovine PMN and PAM. Following opsonization with a particular serum, the % phagocytosis of P. haemolytica was consistently higher for PAM than for PMN. In studies where human phagocytes were compared, PMN phagocytized better than blood monocytes (Horwitz and Silverstein, 1980; Mezzatesta and Rest, 1983), but not better than alveolar macrophages when certain types of organisms were used (Hof et al., 1980). Electron micrographs of phagocytes obtained during the present studies revealed that internalized organisms were being degraded during the assay. Because PMN are apparently more efficient than macrophages at killing microorganisms (Densen and Mandell, 1980), perhaps the lower viable counts used to calculate % phagocytosis in the present studies reflected a higher percentage of killing of internalized bacteria by PMN during the assay.

Neither PAM nor PMN were found to be capable of effective phagocytosis of EO or DO opsonized by heat-inactivated FBS containing no demonstrable antibody to Pasteurella haemolytica. Opsonization of EO with heat-inactivated AL or AC sera enhanced phagocytosis of these organisms by both cell types. In fact, % phagocytosis of EO with AC serum was consistently calculated to be over 100%, presumably due to multiplication of the organisms during the opsonization and phagocytosis incubation periods. Similar bacterial multiplication most likely could not occur with DO opsonized with either antiserum or with EO opsonized with AL serum, due to the bacteriostatic effect of these sera for those organisms (see Chapter IV). This difference in serum bacteriostatic effects could account for the observation that AC serum appeared to be a better opsonin than AL serum for EO, even though AL serum has a higher antibody titer to formalin-killed P. haemolytica organisms as well as to capsular extract (see Chapter IV).

Calculated % phagocytosis was low for DO even after opsonization with AL or AC sera, both of which were shown by agglutination studies to contain antibodies for the DO cell surface. This was an unexpected result because in many previous studies using other organisms, nonencapsulated or decapsulated organisms were found to be phagocytized more efficiently than encapsulated organisms (Maheswaran and Thies, 1979; Horwitz and Silverstein, 1980; Williams et al., 1983). Only one early study suggested that decapsulated Group A Strepococci retained their resistance to phagocytosis by human leukocytes (Rothbard, 1948). The viable count technique used in the present study did not take into account any potential differences in bactericidal activity of sera or intracellular killing rates of internalized encapsulated versus

decapsulated organisms. If, for instance, decapsulation of organisms makes them more susceptible to rapid intracellular killing, low viable counts of cell-associated organisms would be misinterpreted as reduced phagocytosis. Use of radioactively-labelled organisms, whose label would be retained within the phagocytes upon bacterial death, along with viable count techniques would permit differentiation of the two interpretations and might provide a more realistic comparison of % phagocytosis of encapsulated versus decapsulated organisms.

In several studies it has been shown that encapsulated bacteria require both specific antibody and complement for effective opsonization, whereas nonencapsulated organisms can be opsonized by complement alone (Rothbard, 1948; Verbrugh et al., 1979; Horwitz and Silverstein, 1980). Only heat-inactivated sera were used in the present studies because both AL and AC sera were found to be bactericidal for the organisms when they contained complement components (see Chapter IV). Also, in studies using P. multocida, heat-inactivated antisera had been shown to opsonize organisms essentially as well as complement-rich sera (Maheswaran and Thies, 1979; Rush, 1981). However, use of fresh, unheated nonimmune serum as an opsonin for EO and DO should be performed in the future to determine the opsonizing effects of complement on the two cell types in the absence of specific antibody.

Crude IgG fractions of AC and AL sera effectively opsonized EO for phagocytosis by PAM, indicating that the opsonizing capacity of these sera resides in their $(NH_4)_2SO_4$-precipitable fractions. Another indication that the opsonizing capacity of various sera for EO is due to their specific antibodies to the organism is the high positive correlation between opsonizing capacity of the sera and their agglutination titers.

Results of opsonization and phagocytosis assays performed using antisera that were preadsorbed with DO were much like those found for the bactericidal activity of the sera (see Chapter IV). Preadsorption of AL serum did not reduce its FIAX titer to formalin-killed organisms and also only slightly reduced its bactericidal and opsonizing capacities for EO. Preadsorption of AC serum, on the other hand, reduced its FIAX titer to formalin-killed organisms by 47.4% and also drastically reduced its bactericidal and opsonizing capacities by 100% and 89%, respectively.

Results of the experiment utilizing additional viable count samples confirmed that the method used to calculate % phagocytosis in these studies was valid. In future studies, the T_0 counts should be performed both before and after the phagocytosis incubation period. Such an approach would permit detection of differences between samples with respect to ability of the organisms to multiply within the phagocytosis mixture. It does not appear that agglutinated organisms constitute a major component of the high calculated % phagocytosis seen with EO opsonized with AL and AC sera, because the number of intracellular pellet-associated organisms in those samples was 1.0 to 1.5 log units higher than the number of extracellular pellet-associated organisms. In the other samples, the number of intracellular and extracellular pellet-associated organisms was approximately equal. Cohn and Morse (1959) similarly determined after a number of control studies with staphylococci and PMN, that bacterial agglutination was not a major problem in determining phagocytosis and intracellular killing.

Cohn and Morse (1959) also reported that phagocytosis was followed by rapid inactivation of the organisms. Maheswaran et al. (1980) likewise found that P. haemolytica phagocytized by PAM were rapidly

degraded. In the present studies, reduction of the calculated number of intracellular organisms for all samples after differential centrifugation corroborated the electron microscopic evidence indicating degradation of internalized organisms. Nevertheless, the number of viable organisms in incubated suspensions of phagocytes after phagocytosis and differential centrifugation consistently increased. This was most likely due to growth of extracellular organisms. In an *in vitro* study using *P. multocida*, Collins et al. (1983) found that mouse alveolar macrophages only slowly inactivated phagocytized organisms, so that survivors later multiplied extensively. Those workers hypothesized that the surviving organisms probably were not killed because they were cell-associated rather than fully phagocytized. However, in the present studies, growth of extracellular organisms was presumably inhibited by addition of Pen-Strep, but the viable count still increased for half of the samples and was only moderately decreased in others. These data indicate that multiplication of organisms may also be occurring within the phagocytes. During the incubation period a 13-61% decrease in viability of the phagocytes occurred. Organisms sequestered within dead or dying phagocytes could conceivably be protected from the bactericidal effects of antibiotics in the incubation medium; such organisms presumably would be capable of multiplication within this protected environment. However, no correlation could be established between % phagocytosis or % increase in CFU/ml of the phagocyte suspensions and % decrease in phagocyte viability ($p > 0.05$).

Maheswaran et al. (1980) reported previously that at low bacteria/PAM ratios (10:1 or less), there was phagocytosis and killing of *P. haemolytica* but no cytotoxic changes in the PAM. At bacteria/PAM

ratios of 20:1 or higher, however, approximately 10% of the bacteria were not phagocytized and thus replicated and released a substance that was toxic for PAM. Markham and Wilkie (1980) reported that when large numbers of formalin-killed P. haemolytica were added to cultured PAM, there was a positive correlation between increasing uptake of bacteria and loss of PAM from coverslips to which they were attached. Those authors indicated that some property inherent even to killed P. haemolytica accounted for the toxicity. The bacteria/phagocyte ratios used in the majority of the studies described herein was between 10:1 and 20:1. The % decrease in phagocyte viability was more pronounced with PMN than with PAM. Addition of antibiotics or antiserum that neutralizes P. haemolytica cytotoxin to the incubation medium did not prevent phagocyte death. This indicates that killing was not due solely to multiplication and cytotoxin production by extracellular organisms. The decrease in phagocyte viability may have been due to a combination of damage caused by internalized organisms and cytotoxin produced by extracellular, phagocyte-associated organisms. Future studies should include determinations of change in number of viable intracellular organisms versus extracellular ones in phagocyte suspensions following incubation. Such an approach would help to clarify the contribution each makes to phagocyte death.

In conclusion, in these studies phagocytosis of logarithmic-phase P. haemolytica by bovine PAM and PMN was shown to occur in the presence of specific anti-capsular antibodies. Encapsulation of the organisms was not shown to enhance their resistance to phagocytosis. Neither EO nor DO were effectively phagocytized in heat-inactivated FBS. When opsonized by sera containing specific antibodies to the organisms,

EO appeared to be more easily phagocytized than their decapsulated counterparts. Intracellular degradation of organisms was shown by transmission electron microscopy. However, by a viable count technique, intracellular killing of neither EO nor DO could be demonstrated, and a difference in susceptibility between the two types of organisms to killing by phagocytes was not established.

Exposure of bovine phagocytes to P. haemolytica was shown to result in death of both PMN and PAM. PMN were somewhat more susceptible than PAM to the toxic effects of the organisms. EO appeared to be more toxic than DO for PAM, whereas the two types of organisms were equally toxic for PMN. A trend could be seen between % phagocytosis by PAM and the % decrease in PAM viability upon incubation, although significance could not be established mathematically.

Additional experiments need to be performed using viable counts plus accumulation of radioactively-labelled organisms within phagocytes before conclusions should be made about comparisons of phagocytic capability of bovine PMN versus PAM for P. haemolytica or susceptibility of encapsulated versus decapsulated organisms to phagocytosis.

CHAPTER VI

SERUM NEUTRALIZATION OF CYTOTOXIN FROM *PASTEURELLA HAEMOLYTICA* AND COMPARISON OF CYTOTOXIN TO EXTRACTED CAPSULAR MATERIAL

Introduction

Pasteurella haemolytica biotype A serotype 1 has been shown to be associated with severe pneumonic pasteurellosis of feedlot cattle (Lilli, 1974). Recently a cytotoxin which is toxic for bovine pulmonary alveolar macrophages (PAM) and leukocytes (Benson, 1978; Berggren et al., 1981; Kaehler et al., 1980; Markham and Wilkie, 1980) has been demonstrated in supernatant culture fluids obtained during the early logarithmic growth phase of the organism (Baluyut et al., 1981). Because *P. haemolytica* exhibits a specificity for disease production in ruminants as well as a specificity for cytocidal effects on ruminant PAM (Kaehler et al., 1980) and leukocytes (Shewen and Wilkie, 1982), it has been suggested that the cytotoxin may be important in the pathogenesis of bovine pneumonic pasteurellosis (Shewen and Wilkie, 1982).

Partial characterization of *P. haemolytica* cytotoxin has demonstrated it to be a protein (Himmel et al., 1982). Molecular weight determinations have been variable and attempts to purify it have resulted in loss of toxicity (Baluyut et al., 1981; Himmel et al., 1982). An association has not been established between cytotoxin and structural

components of the organism. However, cytotoxin is produced most abundantly during the phase of growth in which the organism is most heavily encapsulated (Corstvet et al., 1982a), suggesting that it may be of capsular origin.

P. haemolytica cytotoxin has been shown to be immunogenic and has been suggested as a potential immunizing agent for cattle (Himmel et al., 1982) in lieu of whole cell bacterins that do not appear to induce resistance (Carter, 1957; Friend et al., 1977; Wilkie et al., 1980). Its toxicity has been shown to be neutralized by antiserum prepared in rabbits (Shewen and Wilkie, 1983a), adult bovine serum (Baluyut et al., 1981; Shewen and Wilkie, 1983b) and bronchoalveolar washings from vaccinated calves (Opuda-Asibo et al., 1983). In the present study an extract of P. haemolytica capsular material was examined for cytotoxicity, and chicken antisera prepared against live P. haemolytica organisms, two preparations of cytotoxin, and a capsular extract (CE) from the bacterium were compared for their ability to neutralize cytotoxin. In addition, sera from several groups of experimental calves were tested for cytotoxin neutralizing capacity and the relationship between this capability and resistance of the animals to an experimental challenge was examined.

Materials and Methods

Organism

The organism used to prepare all of the bacterial products tested was P. haemolytica biotype A serotype 1 which was isolated originally from a feedlot calf (Corstvet et al., 1973). It was maintained in the lyophilized state and was passed periodically in the lungs of 5- to

8-mo old calves, with reisolation and lyophilization between each passage.

Preparation of Capsular Extract

Capsular material was extracted from the organisms as described previously (Gentry et al., 1982). Briefly, the bacteria were suspended in phosphate buffered saline (PBS, 0.01M, pH 7.2) and the suspension was incubated for 1 hr in a shaking water bath at 41° C. The organisms were removed by centrifugation and the extract consisted of the resulting filter sterilized supernatant. For several experiments, CE was concentrated 2 to 10 fold either by lyophilization with reconstitution in smaller volumes or by ultrafiltration (Amicon hollow fiber concentrator, Danvers, MA).

Preparation of Cytotoxins

Three different cytotoxin preparations were utilized. Cytotoxin-S was prepared by the method of Shewen and Wilkie (1982). Briefly, early log-phase organisms were suspended in RPMI 1640 tissue culture medium (GIBCO Laboratories, Grand Island, NY) containing 7% fetal bovine serum (FBS, Biolabs, Inc., Northbrook, IL), and incubated for 1 hr at 37° C. The culture was then centrifuged (6000 x g, 15 sec), filter sterilized, dialyzed against distilled water, refiltered, and lyophilized. Cytotoxin-S-OA was prepared similarly in RPMI 1640 medium containing 6.4 mg/ml ovalbumin (Sigma Chemical Co., St. Louis, MO) in place of the FBS. Cytotoxin-B was prepared by a modification of the method of Baluyut et al. (1981) in which the organisms were grown for 6 hr at

37° C in unsupplemented RPMI 1640 followed by collection and manipulation of the culture supernatant as above.

Bovine Cell Preparation

Polymorphonuclear leukocytes (PMN's) and mononuclear leukocytes were collected from heparinized bovine blood on Ficoll-Paque (Pharmacia, Piscataway, NJ). The mononuclear leukocytes were removed from the interface, washed twice in PBS, and suspended in RPMI 1640 to $5x10^6$ cells/ml. The PMN were separated from the erythrocyte pellet by hypotonic lysis in distilled water followed by addition of double strength PBS to restore tonicity (Mishell and Shiig, 1980). The resulting PMN pellet was washed twice in PBS and resuspended to $5x10^6$ cells/ml. PAM were collected by bronchioalveolar lavage as described previously (Corstvet et al., 1982b). The cells were pelleted by centrifugation of the lavage fluids, washed twice in PBS, and resuspended to $1-5x10^6$ cells/ml in RPMI 1640.

Cytotoxicity Assay

Toxicity of the bacterial products was determined by trypan blue exclusion (Mishell and Shiig, 1980). At 5 min intervals, 200 ul of the product to be tested was added to 100 ul of a bovine cell suspension in a siliconized glass tube. The mixture was incubated for 1-hr at 37° C with mild agitation. At the end of the incubation period, 150 ul of 0.4% trypan blue (GIBCO Laboratories, Grand Island, NY) was added to the mixture and 150-300 cells were examined for viability in a hemacytometer. Quantitation of killing was described as the percent of the total cells remaining viable.

Serum Samples

Chicken antisera were prepared by injection of male Cornish cross chickens with live P. haemolytica organisms, CE, or cytotoxin as described previously (Gentry et al., 1982). Sera were collected also from 5- to 8-mo-old beef calves from four experimental groups: (1) unvaccinated control animals whose sera had low antibody titers to P. haemolytica; (2) unvaccinated animals believed to have been exposed naturally because of high antibody titers to the organism; (3) animals vaccinated either subcutaneously or intradermally with live P. haemolytica type 1; and (4) animals vaccinated either subcutaneously or intradermally with a commercial bacterin of P. haemolytica type 1 and P. multocida type A. Vaccinated calves received two intradermal or subcutaneous injections at 1 wk intervals of either 5 ml of live P. haemolytica organisms ($1x10^9$/ml) or 2 ml of a P. haemolytica and P. multocida bacterin containing an aluminum hydroxide adjuvant (Beecham Laboratories, Bristol, TN). Sera were collected immediately prior to challenge (21 days after the initial vaccination). Antibody titers to formalin-killed P. haemolytica were performed on these sera using a quantitative fluorometric immunoassay (FIAX, International Diagnostic Technology, Santa Clara, CA) as previously described (Confer et al., 1983). All calves were challenged by a transthoracic intrapulmonic injection of $1x10^{10}$ live P. haemolytica in each lung (Panciera and Corstvet, 1984). Lesion scores (LS) were calculated for all of the experimental calves as previously described (Panciera et al., 1984). Briefly, the LS was ascribed to each animal according to specific morphologic parameters of the lung lesions induced by the challenge.

The scores ranged from 0 to 20, with a high LS indicating susceptibility and a low LS indicating resistance to challenge.

Selected sera were adsorbed with formalized decapsulated organisms by incubation for 30 min at 37° C. Organisms were then removed by centrifugation and the sera filter sterilized. The crude IgG fraction of selected sera was isolated by $(NH_4)_2SO_4$ precipitation by a modification of a previously described method (Heide and Schwick, 1978). Briefly, euglobulins were removed by dialysis against distilled water and centrifugation of the resulting precipitate. The supernatant was then treated by dropwise addition of saturated $(NH_4)_2SO_4$. The precipitate formed was removed by centrifugation (800 x g, 15 min), resuspended in PBS, and reprecipitated. The final precipitate was centrifuged, resuspended in PBS to the original serum volume, dialyzed 24 hr against PBS, and resterilized by filtration.

Cytotoxin Neutralization Assay

All neutralization studies were performed using peripheral blood neutrophils as the target cell according to a previously described method (Baluyut et al., 1981). Briefly, lyophilized samples of cytotoxin-S were reconstituted at 1.5 x concentration with PBS. One hundred ul of heat inactivated (56° C for 30 min) serum to be tested was added to 200 ul of cytotoxin and the mixture incubated at 37° C for 10 min immediately prior to utilization of 200 ul of the mixture in a cytotoxicity assay. Daily variations in fragility of the neutrophil preparation and in toxicity of a particular vial of cytotoxin were compensated for by calculation of a protection index (PI) for each serum sample tested by the following formula:

PI = % PMN's killed using control serum - % PMN's killed using test serum / % PMN's killed using control serum

where % of PMN's killed = % alive in control sample using PBS and control serum - % alive in test sample using cytotoxin and test serum

Control serum used was FBS in bovine serum trials and normal chicken serum in chicken serum trials.

In Situ Neutralization Assay

To determine if the serum neutralization shown with pre-formed cytotoxin could also be accomplished as the toxin was being produced, a log phase suspension of P. haemolytica was centrifuged and resuspended in RPMI 1640. The suspension was divided into aliquots of 2.7 ml in sterile centrifuge tubes and 0.3 ml of heat inactivated serum was added to each tube. All tubes were incubated for 1 hr at 37° C in a shaking water bath. The bacteria were then removed by centrifugation followed by filter sterilization and the filtrates were used in the cytotoxicity assay described above.

Statistical Analyses

Cytotoxin neutralization (CN) titers and FIAX titers or LS's were compared by linear regression analysis (Bailey, 1981). Parameter means of treatment groups were compared by Student's t-test.

Results

Comparison of Cytotoxicity of Bacterial Products for Bovine Cells

In the cytotoxicity assay, cytotoxin-S was found to be more toxic for PMN and PAM than either cytotoxin-S-OA or cytotoxin-B (Table 6.1). Therefore, cytotoxin-S was utilized in the remainder of the studies. Comparison of cytotoxin-S to CE for toxicity to PMN's, mononuclear leukocytes, and PAM's indicated that cytotoxin-S was toxic for all cell types, while CE was no more toxic than was a PBS control (Figure 6.1). Concentration of CE (2 to 10 fold) did not enhance cytotoxicity (Figure 6.2).

TABLE 6.1

COMPARISON OF TOXICITY OF CYTOTOXIN PREPARATIONS

Cytotoxin Used	Number of Trials	% of Bovine Cells Viable[a]
Cytotoxin-S[b]	6	29.6 ± 6.3
Cytotoxin-S-OA[c]	4	84.2 ± 4.2
Cytotoxin-B[d]	4	74.5 ± 7.0

[a]Mean ± standard error of the mean.

[b]Prepared in medium containing 7% PBS (Shewen and Wilkie, 1982).

[c]Prepared as for cytotoxin-S in medium containing 6.4 mg/ml ovalbumin.

[d]Prepared in unsupplemented medium (Baluyut et al., 1981).

Figure 6.1. Comparison of Cytotoxicity of Capsular Extract vs. Cytotoxin for Bovine Cells. Bars represent % of cells viable after incubation for 1 hr at 37° C with preparation indicated.

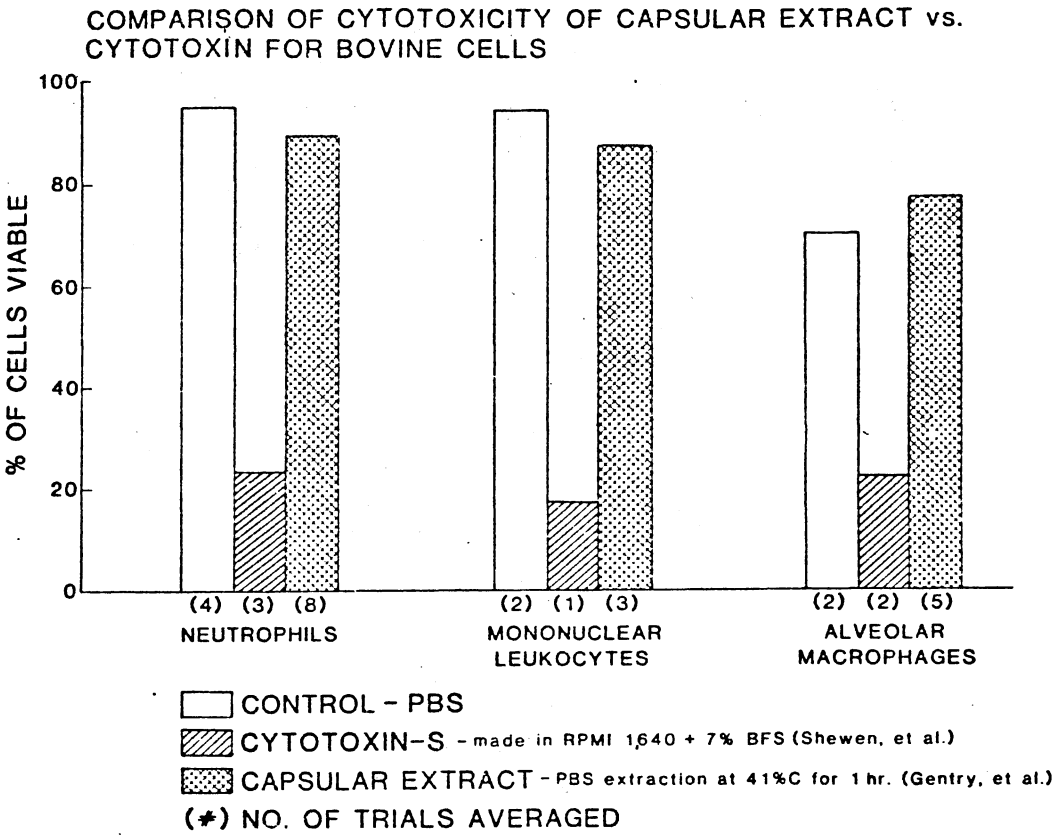
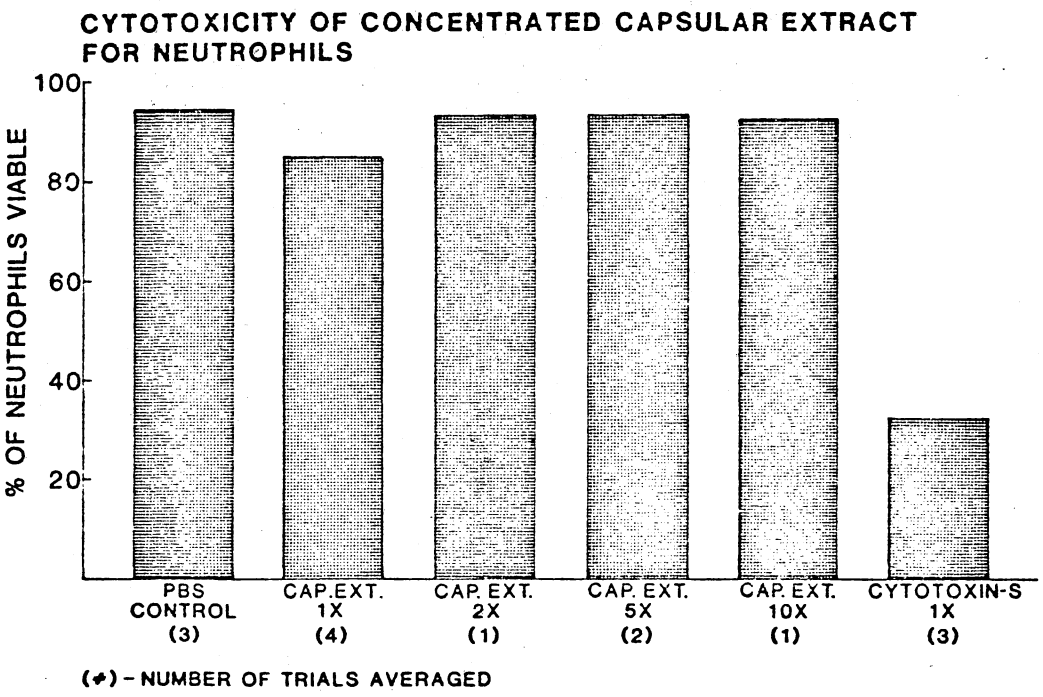

COMPARISON OF CYTOTOXICITY OF CAPSULAR EXTRACT vs. CYTOTOXIN FOR BOVINE CELLS
% OF CELLS VIABLE
100
80
60
40
20
0
(4) (3) (8)
NEUTROPHILS
(2) (1) (3)
MONONUCLEAR LEUKOCYTES
(2) (2) (5)
ALVEOLAR MACROPHAGES
CONTROL - PBS
CYTOTOXIN-S - made in RPMI 1640 + 7% BFS (Shewen, et al.)
CAPSULAR EXTRACT - PBS extraction at 41%C for 1 hr. (Gentry, et al.)
(#) NO. OF TRIALS AVERAGED

Figure 6.2. Cytotoxicity of Concentrated Capsular Extract for Bovine Neutrophils. Bars represent % of cells viable after incubation for 1 hr at 37° C with preparation indicated.


CYTOTOXICITY OF CONCENTRATED CAPSULAR EXTRACT FOR NEUTROPHILS
% OF NEUTROPHILS VIABLE
100
80
60
40
20
PBS CONTROL (3)
CAP.EXT. 1X (4)
CAP. EXT. 2X (1)
CAP. EXT. 5X (2)
CAP. EXT. 10X (1)
CYTOTOXIN-S 1X (3)
(#) - NUMBER OF TRIALS AVERAGED

Because cytotoxin has been shown to be heat labile (Baluyut et al., 1981) an attempt was made to determine if the 41° C treatment used to remove capsular material from the organisms could have destroyed any cytotoxic activity in the CE. Heat treatment of several samples of cytotoxin-S at 41° C for 1 hr resulted in minimal reduction in toxicity (Figure 6.3).

Neutralization of Cytotoxin-S
With Chicken Sera

In order to examine the immunological relationship between CE and cytotoxin, serum neutralization assays of cytotoxin-S were performed. Only anti cytotoxin-B substantially neutralized cytotoxin-S. Antisera to live cells, CE or cytotoxin-S-OA did not neutralize to any extent (Table 6.2).

TABLE 6.2

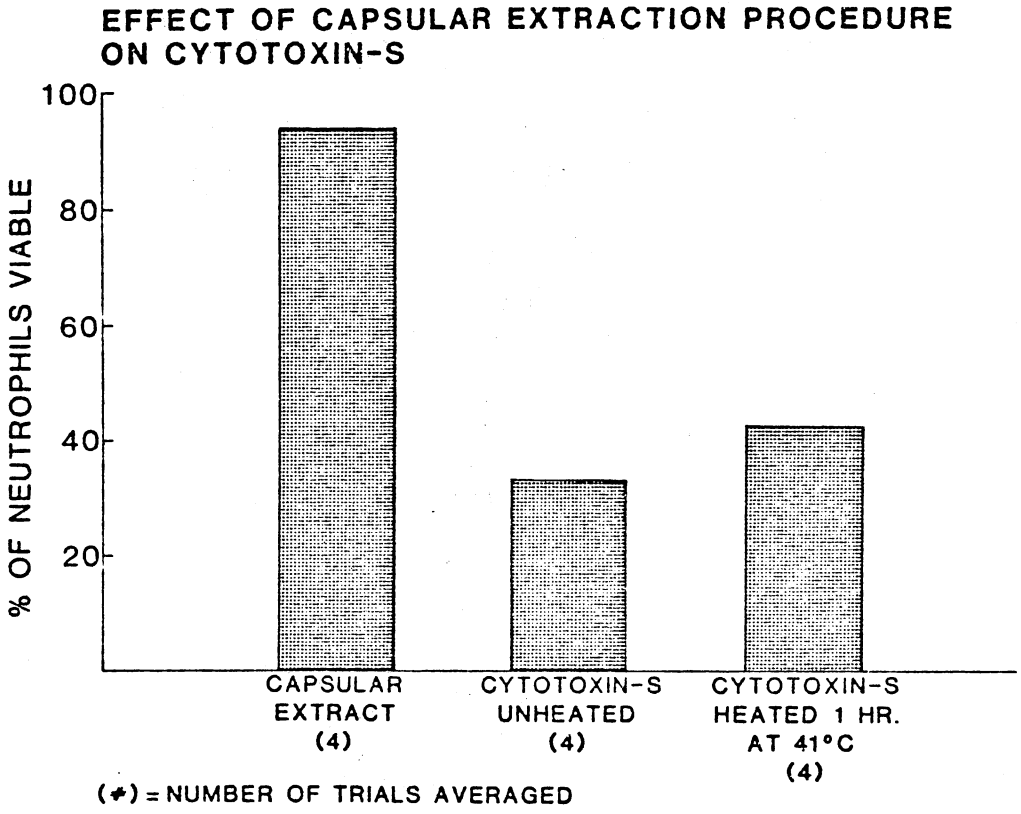
NEUTRALIZATION OF CYTOTOXIN-S WITH CHICKEN ANTISERA

Antiserum Used[a]	Number of Trials	Protection Index[b]
Chicken anti whole organisms	4	0.05 ± 0.04
Chicken anti capsular extract	5	0.03 ± 0.015
Chicken anti cytotoxin-S-OA	3	0.16 ± 0.04
Chicken anti cytotoxin-B	5	0.96 ± 0.025

[a]All antisera were heat-inactivated.

[b]Mean ± standard error of the mean.

Figure 6.3. Effect of Capsular Extraction Procedure on Toxicity of Cytotoxin-S. Bars represent % of cells viable after incubation for 1 hr at 37° C with preparation indicated.


EFFECT OF CAPSULAR EXTRACTION PROCEDURE ON CYTOTOXIN-S
% OF NEUTROPHILS VIABLE
100
80
60
40
20
CAPSULAR EXTRACT (4)
CYTOTOXIN-S UNHEATED (4)
CYTOTOXIN-S HEATED 1 HR. AT 41°C (4)
(#) = NUMBER OF TRIALS AVERAGED

Neutralization of Cytotoxin-S With Bovine Sera

Although the bovine sera used varied considerably in their FIAX titers (0 to 240), all undiluted sera were capable of neutralizing cytotoxin-S as evidenced by PI's of at least 0.85. There was essentially no correlation between the PI's of undiluted sera and FIAX titers or LS's (Figures 6.4 and 6.5). Adsorption of sera with formalized P. haemolytica had essentially no effect on their calculated PI's (Table 6.3). In addition, crude IgG fractions of four sera were as capable of neutralization as were the whole sera from which each was derived (Table 6.3).

TABLE 6.3

COMPARISON OF PROTECTION INDICES CALCULATED FOR CRUDE IgG FRACTIONS AND PREADSORBED BOVINE SERA VERSUS THEIR NATIVE SERA

	PI[a] of Undiluted Serum	PI of IgG Fraction[b]	PI of Adsorbed Serum[c]
Serum A	0.85 ± 0.04	1.0	0.84 ± 0.11
Serum B	0.92 ± 0.05	0.94 ± 0.07	0.86 ± 0.11
Serum C	0.88 ± 0.13	0.92 ± 0.12	-
Serum D	0.80 ± 0.23	0.91 ± 0.13	-

[a]PI = Protection Index. Values represent the mean ± standard deviation.

[b]Crude IgG isolated by $(NH_4)_2SO_4$ precipitation (Heide and Schwick, 1978).

[c]Sera were pre-adsorbed for 30 min with formalized decapsulated organisms.

Figure 6.4. FIAX Titer vs. Protection Index of Undiluted Serum. Regression curve for protection index of undiluted bovine sera vs. FIAX titers.

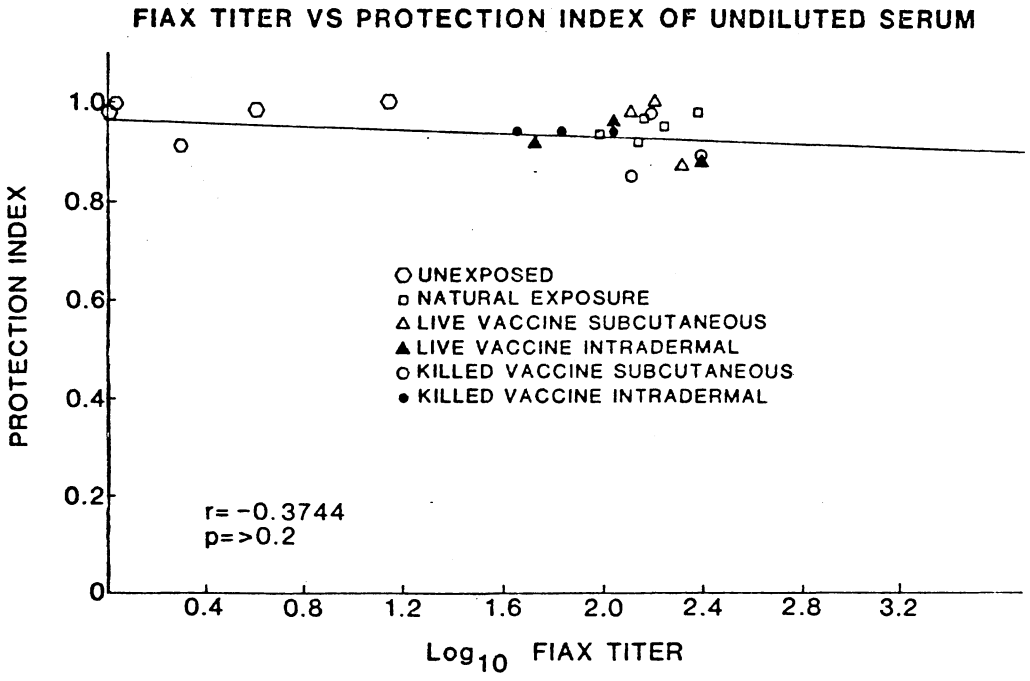
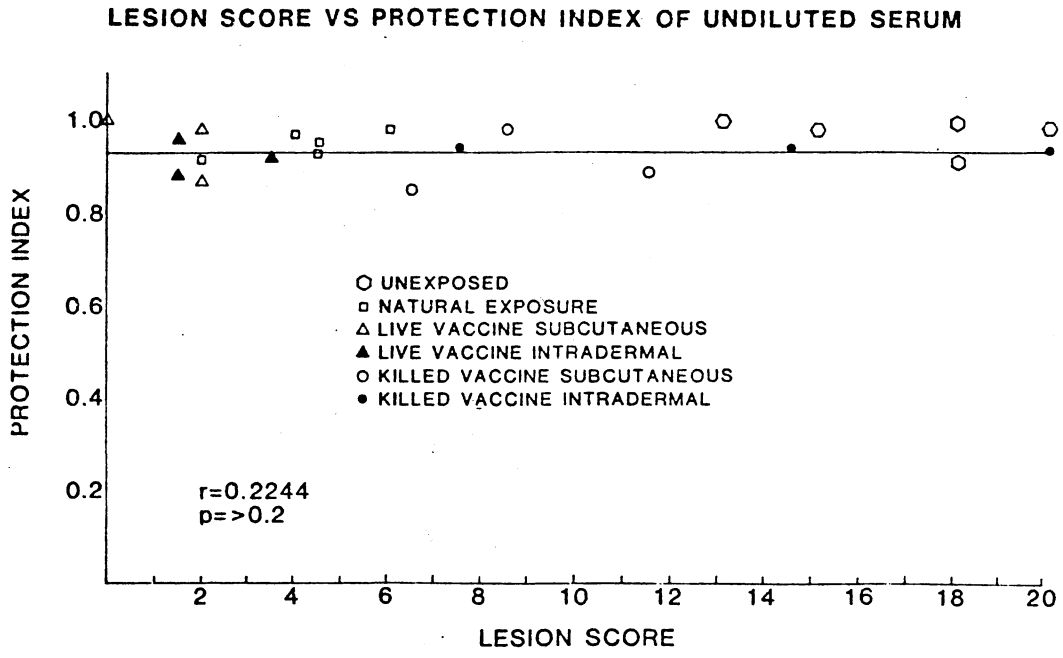

FIAX TITER VS PROTECTION INDEX OF UNDILUTED SERUM
PROTECTION INDEX
1.0
0.8
0.6
0.4
0.2
0
UNEXPOSED
NATURAL EXPOSURE
LIVE VACCINE SUBCUTANEOUS
LIVE VACCINE INTRADERMAL
KILLED VACCINE SUBCUTANEOUS
KILLED VACCINE INTRADERMAL
r= −0.3744
p=>0.2
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
Log10 FIAX TITER

Figure 6.5. Lesion Score vs. Protection Index of Undiluted Serum. Regression curve for protection index of undiluted bovine sera vs. lesion scores.


LESION SCORE VS PROTECTION INDEX OF UNDILUTED SERUM
PROTECTION INDEX
1.0
0.8
0.6
0.4
0.2
UNEXPOSED
NATURAL EXPOSURE
LIVE VACCINE SUBCUTANEOUS
LIVE VACCINE INTRADERMAL
KILLED VACCINE SUBCUTANEOUS
KILLED VACCINE INTRADERMAL
r=0.2244
p=>0.2
2
4
6
8
10
12
14
16
18
20
LESION SCORE

In Situ Neutralization of Cytotoxin-S

As with pre-formed cytotoxin, so chicken anti cytotoxin-B neutralized or prevented *in situ* cytotoxin formation but chicken anti CE serum did not (Table 6.4). All six bovine sera neutralized or prevented cytotoxin formation *in situ* to a degree comparable to that found in the standard neutralization assay (Table 6.4). The culture supernatants were not toxic regardless of whether the antiserum increased or reduced the viable count of the bacteria during incubation (data not shown).

TABLE 6.4

COMPARISON OF PROTECTION INDICES CALCULATED IN *IN SITU* NEUTRALIZATION ASSAY AND STANDARD NEUTRALIZATION ASSAYS

Serum Tested	PI[a] in "in situ" Assay[b]	PI[a] in Standard Assay[c]
Chicken anti Cytotoxin-B	0.90	0.96 ± 0.05
Chicken anti capsular extract	0.15	0.03 ± 0.03
Bovine Serum A	0.91	0.85 ± 0.04
Bovine Serum B	0.99	0.92 ± 0.05
Bovine Serum C	0.88	0.88 ± 0.13
Bovine Serum D	0.71	0.80 ± 0.23
Bovine Serum E	0.76	0.81 ± 0.04
Bovine Serum F	0.92	0.80 ± 0.11

[a]PI = Protection Index.

[b]Represents result from a single trial.

[c]Mean ± standard deviation in 3-5 trials.

Titration of Cytotoxin Neutralizing Capacity for Bovine Sera

Because all of the bovine sera tested were capable of neutralizing cytotoxin, titration curves were performed on two sera to determine if the neutralizing capacities of the sera were comparable. Sera were from an unvaccinated control calf and an animal immunized repeatedly with live P. haemolytica type 1 organisms. At the lowest serum dilutions, both sera were equally capable of neutralizing cytotoxin-S (Figure 6.6). However, serum from the vaccinated animal was capable of neutralization at an 8-fold higher dilution than was serum from the unvaccinated animal. Serum titration curves were then performed for all of the 22 bovine sera included in the original study. The highest dilution of a serum still affording a PI of at least 0.80 was designated as the CN titer for that particular serum. Regression analyses of the CN titers versus FIAX titers and LS's indicated correlation coefficients of 0.54 ($p < 0.01$) and -0.76 ($p < 0.001$), respectively (Figures 6.7 and 6.8).

Comparison of Bovine Treatment Groups

The mean PI for all bovine treatment groups using undiluted serum ranged from 0.91 to 0.98. There were no significant differences in mean PI's among groups ($p > 0.05$). The unexposed group of animals had the highest mean lesion score, the lowest mean FIAX titer, and the lowest mean CN titer. The group of animals considered to have been naturally exposed to the organism had a lower mean lesion score than the unexposed group, and the highest mean FIAX titer and the second highest mean CN titer of all groups (Table 6.5).

Figure 6.6. Effect of Serum Dilution on Cytotoxin Neutralizing Capacity of Bovine Serum. Diluted sera were preincubated for 10 min at 37° C with cytotoxin-S before use in a cytotoxicity assay.

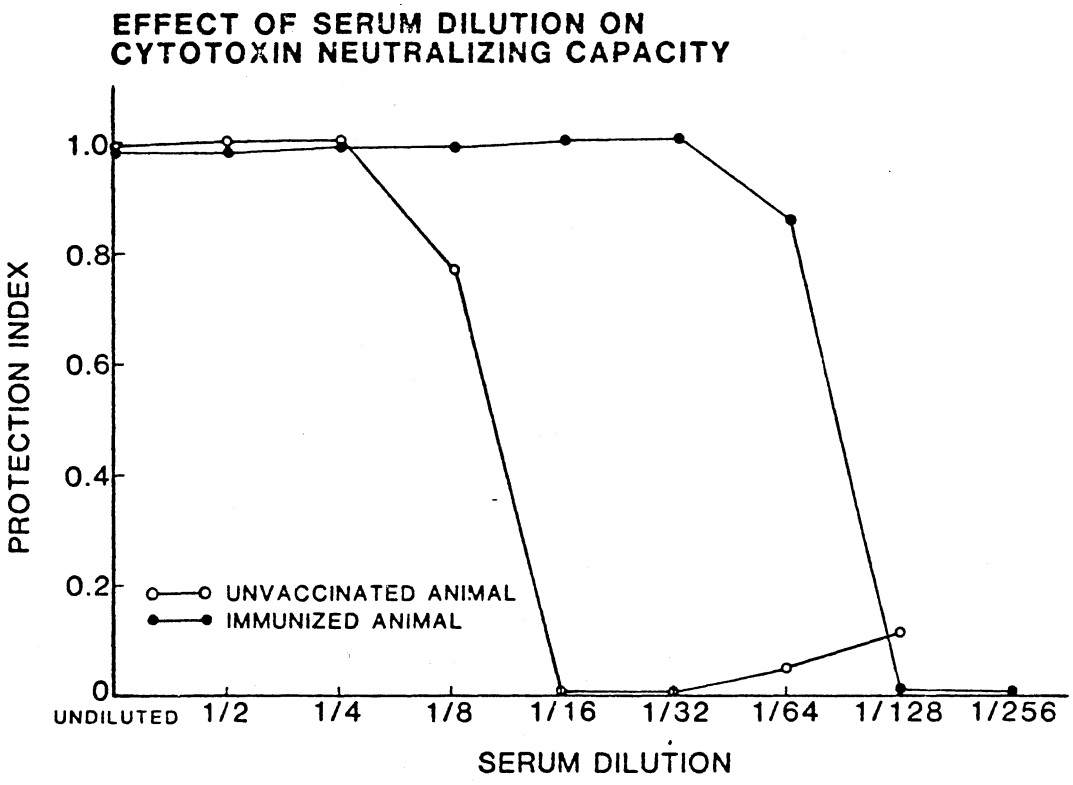

EFFECT OF SERUM DILUTION ON
CYTOTOXIN NEUTRALIZING CAPACITY
PROTECTION INDEX
1.0
0.8
0.6
0.4
0.2
0
UNVACCINATED ANIMAL
IMMUNIZED ANIMAL
UNDILUTED
1/2
1/4
1/8
1/16
1/32
1/64
1/128
1/256
SERUM DILUTION

Figure 6.7. Cytotoxin Neutralization Titer vs. FIAX Titer. Regression curve for cytotoxin neutralization titer of bovine sera vs. FIAX titers.

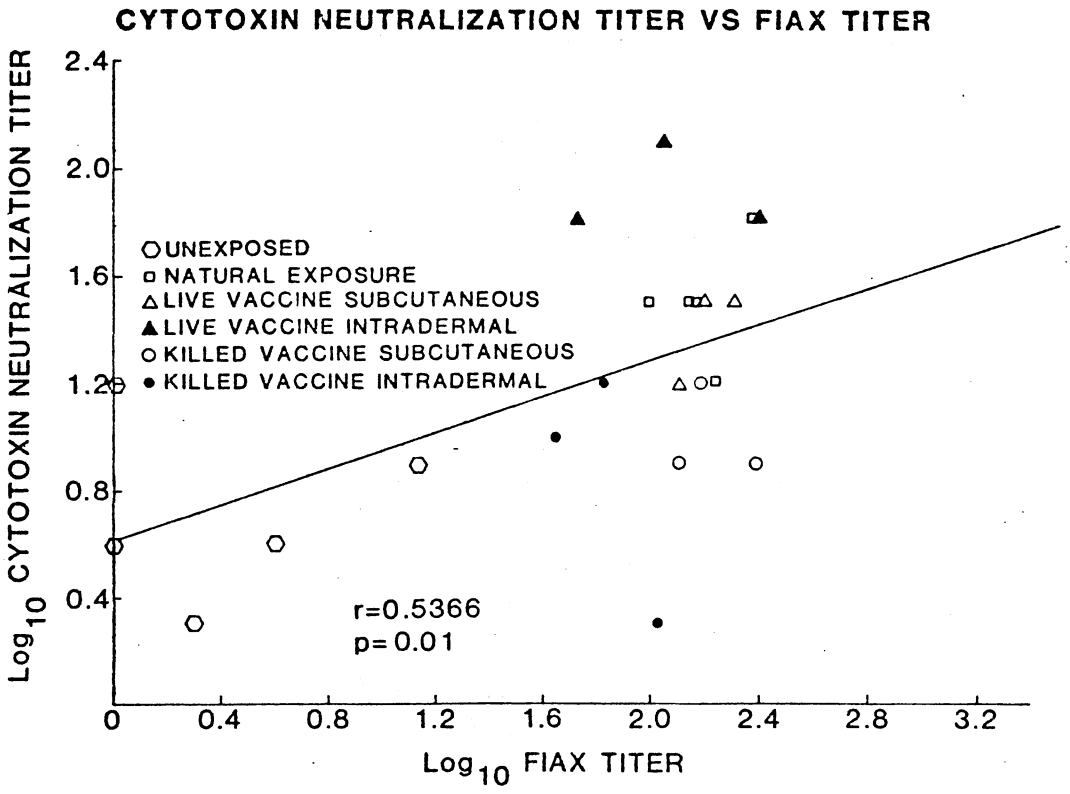

CYTOTOXIN NEUTRALIZATION TITER VS FIAX TITER
Log10 CYTOTOXIN NEUTRALIZATION TITER
2.4
2.0
1.6
1.2
0.8
0.4
UNEXPOSED
NATURAL EXPOSURE
LIVE VACCINE SUBCUTANEOUS
LIVE VACCINE INTRADERMAL
KILLED VACCINE SUBCUTANEOUS
KILLED VACCINE INTRADERMAL
r=0.5366
p=0.01
0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
Log10 FIAX TITER

Figure 6.8. Cytotoxin Neutralization Titer vs. Lesion Score. Regression curve for cytotoxin neutralization titer of bovine sera vs. lesion scores.

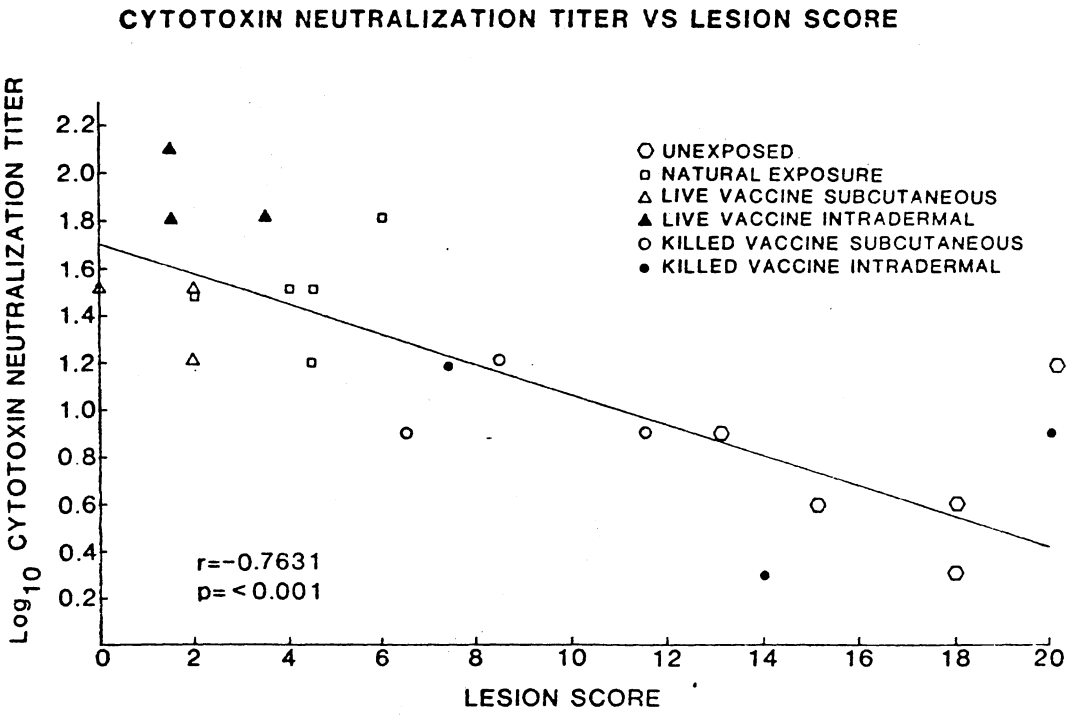

CYTOTOXIN NEUTRALIZATION TITER VS LESION SCORE
Log10 CYTOTOXIN NEUTRALIZATION TITER
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
12
14
16
18
20
LESION SCORE
UNEXPOSED
NATURAL EXPOSURE
LIVE VACCINE SUBCUTANEOUS
LIVE VACCINE INTRADERMAL
KILLED VACCINE SUBCUTANEOUS
KILLED VACCINE INTRADERMAL
r=-0.7631
p=<0.001

TABLE 6.5

PARAMETER MEANS FOR BOVINE TREATMENT GROUPS

Treatment Group	Number of Animals	Lesion Score	FIAX Titer	CN Titer
Unexposed	5	16.8	2.5	5.2
Natural Exposure	5	4.2	148.6	32.2
Live Vaccine	6	1.8	131.8	45.4
Subcutaneous	3	1.3	157.3	25.5
Intradermal	3	2.2	110.5	80.7
Killed Vaccine	6	11.3	105.9	7.9
Subcutaneous	3	8.8	166.0	10.0
Intradermal	3	13.8	67.6	6.3

Comparison of the live vaccine and killed vaccine groups revealed that the live vaccine group had significantly lower LS's ($p < 0.001$) and higher CN titers ($p < 0.02$), while the FIAX titers for the two groups did not differ significantly ($p > 0.10$) (Table 6.5). The mean parameters for the live vaccine group closely paralleled those calculated for the group of naturally exposed animals; only their LS's were significantly lower ($p < 0.05$). At the same level of significance, the killed vaccine group had higher LS's and lower CN titers than the natural exposure group but their FIAX titers were not different. The killed vaccine group's parameter means for lesion score and CN titer did not differ significantly ($p > 0.05$) from those found for the group of unexposed animals. The only parameter significantly different ($p < 0.05$) for these two groups was their FIAX titers.

Comparison of the FIAX titers for the two vaccination routes utilized with killed vaccine revealed that the titers induced by the subcutaneous route were significantly higher ($p < 0.05$) than those induced by the intradermal route (Table 6.5). In contrast, when live vaccine was used, the FIAX titers induced by the two vaccination routes did not differ significantly ($p > 0.05$). However, injection of live organisms by the intradermal route stimulated higher CN titers and higher lesion scores than did injection by the subcutaneous route.

Discussion

The concurrent production of cytotoxin and capsular material by P. haemolytica might suggest that the two products are related. However, in the present studies, we were unable to demonstrate that the extracted capsular material was cytotoxic. This lack of toxicity apparently was not due to a simple dilution effect, because even a 10-fold concentrate of CE was not appreciably toxic. A second possibility, that the 41° C heat treatment used in capsular extraction may have inactivated any cytotoxin present in the CE, does not appear valid either because cytotoxin-S remained toxic after simulated capsular extraction. Finally, efforts to neutralize cytotoxin with antisera from chickens immunized with CE were also unsuccessful, leading to the conclusion that there is not a significant amount of recognizable cytotoxin in CE as prepared in these studies.

Sera from chickens immunized with cytotoxin-S were toxic to bovine PMN's and could not be used in the cytotoxin neutralization assay. Assuming the problem to be due to antibodies formed to the FBS included in cytotoxin-S, we attempted to circumvent the problem

by replacing the FBS with chicken ovalbumin (cytotoxin-S-OA). Apparently little toxin was produced in this medium because the product was only slightly toxic for bovine PMN's and did not stimulate formation of antiserum in chickens which would neutralize cytotoxin-S. Cytotoxin-B, which was prepared by a longer incubation in an unsupplemented medium, was also less toxic for bovine cells than was cytotoxin-S. However, in contrast to cytotoxin-S-OA, it stimulated the formation of antiserum in chickens that was capable of complete neutralization of cytotoxin-S. Perhaps tne prolonged incubation used in preparation of cytotoxin-B allowed for production and subsequent inactivation of cytotoxin, leading to a decrease in toxicity without loss of antigenicity. From these data it appears, then, that FBS is a necessary component of the incubation medium for formation of a maximally toxic product.

Serum from chickens injected with live P. haemolytica organisms did not neutralize cytotoxin-S. This would imply that this organism, which is not a pathogen for chickens, does not multiply to any great extent in this species, or at least that it cannot produce cytotoxin in this unusual environment.

The reason that all bovine sera other than FBS were capable of neutralizing cytotoxin-S in the undiluted state is unclear. Even animals whose FIAX titers indicated that they had not previously been exposed to the organism had PI's of at least 0.80. These data were substantiated by similar results from the in situ neutralization experiment. It may be that the FIAX test, which measures the response to cell surface antigens, was not sensitive enough to detect a minimal immune response. It is also possible for the organism to colonize an

animal's nasal mucosa without invading the submucosa or the remainder of the respiratory tract (Pass and Thomson, 1971). In such a circumstance it is conceivable for the animal's immune system to be stimulated by absorbed soluble toxin that may be produced on the nasal mucosa without making contact with cell surface antigens.

Preadsorption of sera with formalin-killed bacteria did not reduce the PI of the sera. This is further evidence that neutralization of cytotoxin is not mediated by antibodies directed against killed organisms. The lack of correlation between the PI calculated for undiluted sera and the LS's also indicates that the PI of undiluted serum is not a good measure of an animal's resistance to an experimental challenge with the organism.

The $(NH_4)_2SO_4$ precipitation of several antisera and the initial serum titration curves were performed to determine if the serum neutralizing capacity was due to an immunoglobulin or perhaps to another serum factor. The crude IgG fraction of four sera were found to neutralize as well as their native sera. In addition, there was an 8-fold increase in the dilution at which serum from an immunized calf was still capable of neutralizing cytotoxin compared to serum from an unexposed calf, leading to the conclusion that increased neutralizing capacity was due to an immune response.

Prior exposure to live organisms, either naturally or by immunization, as measured by the FIAX titer and CN titer was an important determinant of the animals' ability to resist experimental challenge. Both such exposed groups had significantly higher FIAX and CN titers and lower LS's than the unexposed animals. Vaccination with killed organisms, however, only stimulated an increase in the animals' FIAX

titers. Neither the CN titers nor the LS's of the killed vaccine group were significantly different from those found in the unexposed controls. Therefore, exposure to cell surface proteins on killed organisms did not result in a significant increase in production of cytotoxin neutralizing antibodies and also failed to adequately protect the animals from challenge with live P. haemolytica. These data help to explain the consistent failure of P. haemolytica bacterins to protect cattle adequately from pneumonic pasteurellosis (Carter, 1957; Friend et al., 1977; Wilkie, 1982).

In this study, the sera from calves vaccinated by intradermal injection of live organisms had a greater capacity to neutralize cytotoxin than did sera from calves vaccinated by subcutaneous injection. The lesion scores, however, were somewhat lower for the animals vaccinated subcutaneously. The use of such small treatment groups makes it difficult to compare the two injection routes adequately. However, the differences shown bear further examination.

In conclusion, although the capsular material and cytotoxin of P. haemolytica are both produced most abundantly during the early logarithmic growth phase of the organism, these studies failed to show any toxicological or immunological relationship between the two products. All undiluted bovine sera tested, other than FBS, were capable of neutralizing cytotoxin. Sera varied, however, in their CN titers, and the animals' capability to resist an experimental P. haemolytica challenge closely correlated with these titers. Statistical evaluation of the animals' serum antibody levels to cell surface antigens as reflected in their FIAX titers did not predict this difference. These results thus lend support to the hypothesis

that protection against experimental challenge with P. haemolytica, type 1 may require an immune response to cytotoxin (Wilkie, 1982).

CHAPTER VII

SUMMARY AND CONCLUSIONS

The studies described herein were undertaken to examine the relationship of capsular material on Pasteurella haemolytica to the organism itself and to the organism's involvement in the bovine disease known as shipping fever or pneumonic pasteurellosis.

Chapter II was concerned with microscopic demonstration of the capsular material by a fluorescent antibody and several capsular staining techniques. Wide capsules were demonstrated on organisms from 2- to 6-hr cultures. The amount of capsular material diminished gradually as the culture aged, until little capsular material remained on organisms from cultures over 16 hr old. For capsule demonstration, Maneval's stain was determined to be superior to the other staining techniques.

In Chapter III, it was determined that capsular material could be removed from the organisms by incubating them at several different temperatures in either distilled water or phosphate buffered saline (PBS). Criteria used to determine capsular removal included change in antibody-mediated agglutinability of the organisms and disappearance of an antigenic component by the fluorescent-antibody test and the agar-gel diffusion technique. Incubation of a PBS-suspension at 41° C for 1 hr was determined to be the method of choice for removing capsules with a minimum decrease in culture viability. That method

was then used in subsequent experiments to provide comparable populations of encapsulated and decapsulated organisms, as well as capsular extracts. Extracts consisted of the PBS supernatants from centrifuged and filter-sterilized decapsulated bacterial suspensions.

Chapter IV dealt with the interactions of encapsulated organisms (EO) and decapsulated organisms (DO) with bovine sera. DO were more easily agglutinated than EO by antiserum prepared against intact organisms, whereas EO were more easily agglutinated than DO by antiserum prepared against a capsular extract from the bacterium. DO were found to be more sensitive than EO to the bactericidal action of bovine sera. High antibody titers, particularly to capsular material, were apparently needed for serum killing of EO, whereas DO were readily killed by sera even from animals with low antibody titers. Heat-inactivation of the sera reduced the rate and the percentage of killing of both EO and DO. Addition of a fresh complement source partially restored the bactericidal activity of heat-inactivated sera.

In Chapter V, EO and DO were also compared as to their interactions with bovine phagocytes. A viable counting technique was used to determine the % phagocytosis of the two types of organisms after opsonization with various heat-inactivated bovine sera. Neither EO nor DO were effectively phagocytized after opsonization by fetal bovine serum. After opsonization with either anti-live (AL) or anti-capsular (AC) serum, however, both types of organisms were phagocytized by bovine polymorphonuclear leukocytes (PMN) or pulmonary alveolar macrophages (PAM). The calculated % phagocytosis for EO was 2 and 10 times higher than that calculated for DO after opsonization with AL or AC serum, respectively. Opsonizing capacity was shown to reside in

the IgG fractions of the sera. Intracellular killing of the organisms was shown to occur by transmission electron microscopy, and by a reduction in the calculated number of intracellular organisms during the assay. However, intracellular killing could not be demonstrated for either type of organism by incubation of phagocytes after separation from unphagocytized bacteria by a differential centrifugation technique. Exposure of bovine PMN or PAM to either EO or DO resulted in death of the phagocytes, with PMN being somewhat more susceptible than PAM to the organisms' toxic effects. EO were more toxic than DO for PAM.

In Chapter VI, P. haemolytica capsular extract was compared to several preparations of cytotoxin from the organism. These experiments failed to show any immunological or toxicological relationships between the two products. In addition, a study was performed of the neutralization of P. haemolytica cytotoxin by bovine sera from a number of animals with varying degrees and types of immune responses to P. haemolytica. All undiluted sera tested, other than fetal bovine serum, were capable of neutralizing cytotoxin, regardless of their antibody titer against P. haemolytica. However, the cytotoxin neutralizing capacities of the sera, as determined by titration curves, differed according to the type of prior exposure of the animals to the organism. Animals exposed to live organisms, either naturally or by immunization, had significantly higher cytotoxin neutralizing titers and significantly greater resistance to experimental disease than animals which were unexposed or vaccinated with bacterins.

From the results determined in these studies, the following conclusions were drawn:

1. Studies of capsular material on P. haemolytica should be carried out with early log-phase cultures in order to obtain maximally encapsulated organisms.

2. Maneval's capsular staining method is a rapid and reliable technique for demonstration of P. haemolytica capsular material.

3. Viable decapsulated P. haemolytica may be prepared by incubation of PBS suspensions of the organisms at 41° C for 1 hr.

4. Capsular extracts of P. haemolytica prepared by the method above contain both protein and carbohydrate. They are immunogenic for chickens and cattle, but are not toxic to bovine phagocytes.

5. Encapsulated P. haemolytica are generally more resistant to serum agglutination than are nonencapsulated or decapsulated organisms.

6. Capsular material on P. haemolytica from logarithmic-phase cultures appears to enhance resistance of the organisms to the bactericidal action of bovine serum. Complement is necessary for maximum bactericidal activity against either encapsulated or decapsulated organisms.

7. Neither encapsulated nor decapsulated P. haemolytica are phagocytized by bovine PMN or PAM in the absence of specific antibody.

8. Encapsulated P. haemolytica are phagocytized effectively by bovine phagocytes after opsonization with heat-inactivated antisera containing anti-capsular antibodies. Encapsulation does not appear to enhance resistance of the organisms to phagocytosis, at least in the presence of anti-capsular antibodies.

9. Intracellular killing of P. haemolytica occurs after phagocytosis by bovine PAM.

10. Both encapsulated and decapsulated P. haemolytica are toxic to bovine phagocytes.

11. The capacity of an animal's serum to neutralize P. haemolytica cytotoxin may be one important determinant of the animal's ability to resist experimental challenge with the organism.

12. Induction of significant serum cytotoxin neutralization titers in cattle depends on exposure of the animals to live, metabolically active organisms.

BIBLIOGRAPHY

Abinanti, F. R. 1968. Future requirements for prevention and control of bovine respiratory diseases in the United States. J. Am. Vet. Med. Ass. 152:934-937.

Alexander, J. W. and R. A. Good. 1968. Effect of antibiotics on the bactericidal activity of human leukocytes. J. Lab. Clin. Med. 71:971-982.

Amstutz, H. E., L. A. Horstman, and R. L. Morter. 1981. Clinical evaluation of the efficacy of Haemophilus somnus and Pasteurella sp. bacterins. The Bov. Practitioner 16:106-108.

Anwar, H., M. R. W. Brown, and P. A. Lambert. 1983. Effect of nutrient depletion on sensitivity of Pseudomonas cepacia to phagocytosis and serum bactericidal activity at different temperatures. J. Gen. Microbiol. 129:2021-2027.

Atkinson, J. P. and M. M. Frank. 1980. Complement pp. 219-271 in "Clinical Immunology," Vol. 1, ed. C. W. Parker. Philadelphia: W. B. Saunders Co.

Bailey, N. T. J. 1981. Statistical methods in biology. 2nd ed. New York: John Wiley and Sons, Inc., 43-51; 133-145.

Baldwin, D. E., R. G. Marshall, and G. E. Wessman. 1967. Experimental infection of calves with myxovirus parainfluenza-3 and Pasteurella haemolytica. Am. J. Vet. Res. 28:1773-1782.

Baluyut, C. S., R. R. Simonson, W. J. Bemrick, and S. K. Maheswaran. 1981. Interaction of Pasteurella haemolytica with bovine neutrophils: Identification and partial characterization of a cytotoxin. Am. J. Vet. Res. 42:1920-1926.

Bennett, B. W. 1982. Efficacy of Pasteurella bacterins for yearling feedlot cattle. Bov. Practice 3:26-30.

Benson, M. L., R. C. Thomson, and V. E. O. Valli. 1978. The bovine alveolar macrophages. II. In vitro studies with Pasteurella haemolytica. Can. J. Comp. Med. 42:368-369.

Berggren, K. A., C. S. Baluyut, R. R. Simonson, W. J. Bemrick, and S. K. Maheswaran. 1981. Cytotoxic effects of Pasteurella haemolytica on bovine neutrophils. Am. J. Vet. Res. 42:1383-1388.

Biberstein, E. L. and M. G. Gills. 1962. The relation of the antigenic types to the A and T types of Pasteurella haemolytica. J. Comp. Path. 72:316-320.

Biberstein, E. L., M. Gills, and H. Knight. 1960. Serological types of Pasteurella haemolytica. Cornell Vet. 50:283-300.

Biberstein, E. L., M. E. Meyer, and P. C. Kennedy. 1958. Colonial variation of Pasteurella haemolytica isolated from sheep. J. Bacteriol. 76:445-452.

Biberstein, E. L. and D. A. Thompson. 1965. Type specificity of immunity to Pasteurella haemolytica infection in mice. J. Comp. Path. 75: 331-337.

Biberstein, E. L. and D. A. Thompson. 1966. Epidemiological studies on Pasteurella haemolytica in sheep. J. Comp. Path. 76:83-94.

Bjornson, A. B. and J. G. Michael. 1970. Biological activities of rabbit immunoglobulin M and immunoglobulin G antibodies to Pseudomonas aeruginosa. Infect. and Immun. 2: 453-461.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Branefors, P. and T. Dahlberg. 1980. Serum bactericidal effect on capsulated and non-capsulated Haemophilus influenzae. Acta. Path. Microbiol. Scand. Sect. C. 88:47-56.

Buckley, J. S. and W. S. Gochenour. 1924. Immunization against hemorrhagic septicemia. J. Am. Vet. Met. Ass. 66:308-311.

Bukantz, S. C., A. Cooper, and J. G. M. Bullowa. 1941. The elaboration of soluble capsular polysaccharide by Pneumococcus III in relation to growth phases in vitro. J. Bacteriol. 42:29-39.

Butt, E. M., C. W. Bonynge, and R. L. Joyce. 1936. The demonstration of capsules about haemolytic streptococci with India ink or azo blue. J. Infect. Dis., 58:5-9.

Campbell, S. G., S. S. Mtero, C. J. Niderbuhl, and F. M. Collins. 1983. Fate of Pasteurella haemolytica in conventionally raised and germfree mice. Infect. and Immun. 39:202-207.

Carter, G. R. 1956a. A serological study of Pasteurella haemolytica. Can. J. Microbiol. 2:483-488.

Carter, G. R. 1956b. Studies on pneumonia of cattle I. Experimental infection of calves with Pasteurella haemolytica. Can. J. Comp. Med. 20:374-380.

Carter, G. R. 1957. A bacterin for the prevention of shipping fever in Canada. Vet. Med. 52:254-255.

Carter, G. R. 1968. Pasteurellosis: Pasteurella multocida and Pasteurella haemolytica. Adv. Vet. Sci. 11:321-379.

Cavanaugh, D. C. and R. Randall. 1959. The role of multiplication of Pasteurella pestis in mononuclear phagocytes in the pathogenesis of flea borne plague. J. Immunol. 83:348-363.

Ceppellini, R. and M. Landy. 1963. Suppression of blood group agglutinability of human erythrocytes by certain bacterial polysaccharides. J. Expl. Med. 117:321-338.

Chambers, V. C. 1973. The use of ruthenium red in an electron microscope study of cytophagocytosis. J. Cell Biol. 57:874-878.

Cline, M. J. and M. C. Territo. 1980. Phagocytosis pp. 298-313 in "Clinical Immunology", Vol. 1, ed. C. W. Parker. Philadelphia: W. B. Saunders Co.

Cohn, Z. A. 1963. The fate of bacteria within phagocytic cells. I. The degradation of isotopically labelled bacteria by polymorphonuclear leukocytes and macrophages. J. Exp. Med. 117:27-42.

Cohn, Z. A. and S. I. Morse. 1959. Interactions between rabbit polymorphonuclear leukocytes and staphylococci. J. Exp. Med. 110: 419-443.

Cohn, Z. A. and S. I. Morse. 1960. Functional and metabolic properties of polymorphonuclear leucocytes. II. The Influence of a lipopolysaccharide endotoxin. J. Exp. Med. 111:688-704.

Cole, P. and J. Brostoff. 1975. Intracellular killing of Listeria monocytogenes by activated macrophages (mackaness system) is due to antibiotic. Nature 256:515-517.

Collier, J. R. 1968. Pasteurellae in bovine respiratory disease. J. Am. Vet. Med. Ass. 152:824-828.

Collier, J. R., T. L. Chew, M. M. Benjamin, and A. W. Deem. 1960. The combined effect of infectious bovine rhinotracheitis virus and Pasteurella haemolytica on cattle. Am. J. Vet. Res. 21:195-198.

Collins, F. M., C. J. Niederbuhl, and S. G. Campbell. 1983. Bactericidal activity of alveolar and peritoneal macrophages exposed in vitro to three strains of Pasteurella multocida. Infect. and Immun. 39:779-784.

Confer, A. W., R. J. Panciera, R. E. Corstvet, J. A. Rummage, and R. W. Fulton. 1984a. Bovine pneumonic pasteurellosis: Effect of culture age of Pasteurella haemolytica used as a live vaccine. Am. J. Vet. Res. in press.

Confer, A. W., R. J. Panciera, R. W. Fulton, M. J. Gentry, and J. A. Rummage. 1984b. Effect of vaccination with live or killed Pasteurella haemolytica on resistance to experimental bovine pneumonic pasteurellosis. Am. J. Vet. Res. submitted for publication.

Confer, A. W., J. C. Wright, J. M. Cummins, R. J. Panciera, and R. E. Corstvet. 1983. Use of a fluorometric immunoassay to determine antibody response to Pasteurella haemolytica in vaccinated and nonvaccinated feedlot cattle. J. Clin. Microbiol. 18:866-871.

Corstvet, R. E., M. J. Gentry, P. R. Newman, J. A. Rummage, and A. W. Confer. 1982a. Demonstration of age-dependent capsular material on Pasteurella haemolytica serotype 1. J. Clin. Microbiol. 16:1123-1126.

Corstvet, R. E., R. J. Panciera, and P. Newman. 1978. Vaccination of calves with Pasteurella multocida and Pasteurella haemolytica. Proc. Am. Assoc. Vet. Lab. Diagn. 21:67-90.

Corstvet, R. E., R. J. Panciera, H. B. Rinker, B. L. Starks, and C. Howard. 1973. Survey of tracheas of feedlot cattle for Haemophilus somnus and other selected bacteria. J. Am. Vet. Med. Ass. 163:870-873.

Corstvet, R. E., J. A. Rummage, and J. T. Homer. 1982b. Recovery of pulmonary alveolar macrophages from nonanesthetized calves. Am. J. Vet. Res. 43:2253-2254.

Corstvet, R. E. and W. W. Sadler. 1964. Use of the fluorescent antibody technique in the diagnosis of certain avian diseases. Poultry Sci. 43:1280-1288.

Davies, W. A. 1982. Kinetics of killing Listeria monocytogenes by macrophages: Correlation of ^{3}H-DNA release from labelled bacteria and changes in numbers of viable organisms by mathematical model. J. Reticuloendo. Soc. 32:461-476.

Davis, S. D. and R. J. Wedgwood. 1965. Kinetics of the bactericidal action of normal serum on gram-negative bacteria. J. Immunol. 95:75-79.

Densen, P. and G. L. Mandell. 1980. Phagocyte strategy vs. microbial tactics. Rev. Infect. Dis. 2:817-838.

Dische, Z. 1961. Experimental Immunochemistry. 2nd ed. Springfield, IL: Charles C. Thomas.

Duguid, J. P. and J. F. Wilkinson. 1953. The influence of cultural conditions on polysaccharide production by Aerobacter aerogenes. J. Gen. Microbiol. 9:174-189.

Dyer, R. M. 1982. The bovine respiratory disease complex: A complex interaction of host, environmental, and infectious factors. The Comp. on Cont. Educ. for Pract. Veterinarian. 4:S296-S307.

Edwards, B. L. 1959. A note on hemorrhagic septicemia in neonatal pigs. Vet. Rec. 71:208.

Elsbach, P. 1980. Degradation of microorganisms by phagocytic cells. Rev. Infect. Dis. 2:106-128.

Evans, H. B. and P. W. Wells. 1979. A mouse model of Pasteurella haemolytica infection and its use in assessment of the efficacy of P. haemolytica vaccines. Res. Vet. Sci. 27:213-217.

Farley, H. 1932. An epizootiological study of shipping fever in Kansas. J. Am. Vet. Med. Ass. 80:165-172.

Fierer, J. and F. Finley. 1979. Lethal effect of complement and lysozyme on polymyxin-treated, serum-resistant gram-negative bacilli. J. Infect. Dis. 140:581-589.

Fierer, J., F. Finley, and A. I. Braude. 1972. A plaque assay on agar for detection of gram-negative bacilli sensitive to complement. J. Immunol. 109:1156-1158.

Fierer, J., F. Finley, and A. I. Braude. 1974. Release of ^{51}Cr-endotoxin from bacteria as an assay of serum bactericidal activity. J. Immunol. 112:2184-2192.

Filion, L. G., R. L. McGuire, and L. A. Babiuk. 1983. Nonspecific suppressive effect of bovine herpes virus type 1 on bovine leukocyte functions. Infect. and Immun. 42:106-112.

Finch, J. E. and M. R. W. Brown. 1978. Effect of growth environment on Pseudomonas aeruginosa killing by rabbit polymorphonuclear leukocytes and cationic proteins. Infect. Immun. 20:340-346.

Flügge. 1886. Cited in Gillespie and Timoney, "Hagan and Bruner's Infectious Diseases of Domestic Animals," 7th ed. Ithaca, NY: Cornell University Press, 1981.

Frank, G. A. and G. E. Wessman. 1978. Rapid plate agglutination procedure for serotyping Pasteurella haemolytica. J. Clin. Microbiol. 7:142-145.

Friedlander, A. M. 1975. DNA release as a direct measure of microbial killing. I. Serum bactericidal activity. J. Immunol. 115:1404-1408.

Friedlander, A. M. 1978. DNA release as a direct measure of microbial killing by phagocytes. Infect. and Immun. 22:148-154.

Friend, S. C. E, B. N. Wilkie, R. G. Thomson, and D. A. Barnum. 1977. Bovine pneumonic pasteurellosis: Experimental induction in vaccinated and nonvaccinated calves. Can. J. Comp. Med. 41:77-83.

Gentry, M. J. and A. W. Confer. 1983. Serum neutralization of Pasteurella haemolytica cytotoxin. Abst. 179, Proc. 64th Annual Meeting, Conf. Res. Workers An. Dis., Chicago, Ill.

Gentry, M. J., R. E. Corstvet, and R. J. Panciera. 1982. Extraction of capsular material from Pasteurella haemolytica. Am. J. Vet. Res. 43:2070-2073.

Gillespie, J. H. and J. F. Timoney. 1981. "Hagan and Bruner's Infectious Diseases of Domestic Animals." 7th ed. Ithaca, NY: Cornell University Press.

Gilmour, N. J. L., W. B. Martin, J. M. Sharp, D. A. Thompson, and P. W. Wells. 1979. The development of vaccines against pneumonic Pasteurellosis in sheep. Vet. Rec. 111:15.

Gilmour, N. J. L., W. B. Martin, J. M. Sharp, D. A. Thompson, P. W. Wells, and W. Donachie. 1983. Experimental immunization of lambs against pneumonic pasteurellosis. Res. Vet. Sci. 35:80-86.

Glynn, A. A. and C. J. Howard. 1970. The sensitivity to complement of strains of Escherichia coli related to their K antigens. Immunol. 18:331-346.

Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein. 1969. Human immunity to the meningococcus II. Development of natural immunity. J. Exp. Med. 129:1327-1348.

Goto, M. and C. Itakura. 1975. Lesions of central nervous system in a calf due to a Pasteurella organism. Jap. J. Vet. Sci. 37:303-306.

Griffin, F. M., Jr., J. A. Griffin, and S. C. Silverstein. 1976. Studies on the mechanism of phagocytosis. II. The interaction of macrophages with anti-immunoglobulin IgG-soaked bone marrow-derived lymphocytes. J. Exp. Med. 144:788-809.

Hamdy, A. H. 1968. Role of mycoplasma in bovine respiratory infection. J. Am. Vet. Med. Ass. 152:829-832.

Hamdy, A. H., C. Gale, and N. B. King. 1958. Studies of shipping fever of cattle. II. Isolation of pleuropneumonia-like organisms. Am. J. Vet. Res. 19:818-821.

Hamdy, A. H., N. B. King, and A. L. Trapp. 1965. Attempted immunization of cattle against shipping fever: A field trial. Am. J. Vet. Res. 26:897-902.

Hamdy, A. H. and A. L. Trapp. 1964. Immunization of cattle against shipping fever: Experimental exposure. Am. J. Vet. Res. 25: 1648-1652.

Hardenbergh, J. B. and F. Boerner. 1918. Hemorrhagic septicemia and its control in Pennsylvania. J. Am. Vet. Med. Ass. 53:482-493.

Heddleston, K. L., J. E. Gallagher, and P. A. Rebers. 1972. Fowl cholera: Gel diffusion precipitin test for serotyping Pasteurella multocida from avian species. Avian Dis. 16:925-936.

Heide, K. and H. G. Schwick. 1978. Salt Fractionation of Immunoglobulin pp. 7.2-7.3 in "Handbook of Experimental Immunology." ed. D. M. Weir. Oxford, England: Blackwell Scientific Publications.

Hill, A. W., D. J. S. Heneghan, and M. R. Williams. 1983. The opsonic activity of bovine milk whey for the phagocytosis and killing of encapsulated and non-encapsulated Escherichia coli. Vet. Microbiol. 8:293-300.

Himmel, M. E., M. D. Yates, L. H. Lauerman, and P. G. Squire. 1982. Purification and partial characterization of a macrophage cytotoxin from Pasteurella haemolytica. Am. J. Vet. Res. 43:764-767.

Hiss, P. H. 1905. A contribution to the physiological differentiation of Pneumococcus and Streptococcus, and to methods of staining capsules. J. Exp. Med. 6:317-345.

Hoerlein, A. B., S. P. Saxena, and M. E. Mansfield. 1961. Studies on shipping fever of cattle. II. Prevalence of Pasteurella species in nasal secretion from normal calves and calves with shipping fever. Am. J. Vet. Res. 22:470-472.

Hof, D. G., J. E. Repine, P. K. Peterson, and J. R. Hoidal. 1980. Phagocytosis by human alveolar macrophages and neutrophils: Qualitative differences in the opsonic requirements for uptake of Staphylococcus aureus and Streptococcus pneumoniae in vitro. Am. Res. Resp. Dis. 121:65-71.

Hofing, G. L., H. G. Rush, A. R. Petkus, and J. C. Glorioso. 1979. In vitro killing of Pasteurella multocida: The effect of rabbit granulocyte and specific antibody source. Am. J. Vet. Res. 40: 679-683.

Horwitz, M. A. and S. C. Silverstein. 1980. Influence of the Escherichia coli capsule on complement fixation and on phagocytosis and killing by human phagocytes. J. Clin. Invest. 65:82-94.

Howie, J. W. and J. Kirkpatrick. 1934. Observations on bacterial capsules as demonstrated by a simple method. J. Path. 39:165-169.

Hsu, H. S. and A. S. Radcliffe. 1968. Interactions between macrophages of guinea pigs and salmonellae. I. Fate of Salmonella typhimurium within macrophages of normal guinea pigs. J. Bacteriol. 96:191-197.

Irwin, M. R., S. McConnell, J. D. Coleman, and G. E. Wilcox. 1979. Bovine respiratory disease complex: A comparison of potential predisposing and etiological factors in Australia and the United States. J. Am. Vet. Med. Ass. 175:1095-1099.

Jakab, G. J., G. A. Warr, and P. L. Sannes. 1980. Alveolar macrophage ingestion and phagosome-lysosome fusion defect associated with virus pneumonia. Infect. and Immun. 27:960-968.

Jasmin, A. M. 1945. An improved staining method for demonstrating bacterial capsules with particular reference to Pasteurella. J. Bacteriol. 50:361-363.

Jenson, R. 1968. Scope of the problem of bovine respiratory disease in beef cattle. J. Am. Vet. Med. Ass. 152:720-723.

Jenson, R., R. E. Pierson, M. Braddy, D. A. Saari, L. H. Lauerman, J. J. England, H. Keyvanfar, J. R. Collier, D. P. Horton, A. E. McChesney, A. Benitez, and R. M. Christie. 1976. Shipping fever pneumonia in yearling feedlot cattle. J. Am. Vet. Med. Ass. 169: 500-507.

Jericho, K. W. F. and E. V. Langford. 1978. Pneumonia in calves produced with aerosols of bovine herpes virus 1 and Pasteurella haemolytica. Can. J. Comp. Med. 42:269-277.

Jericho, K. W. F., S. E. Magwood, and P. H. G. Stockdale. 1976. Prevention of experimental bovine pneumonic pasteurellosis by exposure to IBR virus. Can. Vet. J. 17:194-195.

Jones, F. S. 1921. A study of Bacillus bovisepticus. J. Exp. Med. 34:561-577.

Jorgenson, C. E. 1925. Some studies of Pasteurella boviseptica. Cornell Vet. 15:295-302.

Jubb, K. V. F. and P. C. Kennedy. 1970. Pathology of Domestic Animals. Vol. 1, 2nd ed. New York: Academic Press.

Kaehler, K. L., R. J. F. Markham, C. C. Muscoplat, and D. W. Johnson. 1980a. Evidence of cytocidal effects of Pasteurella haemolytica on bovine peripheral blood mononuclear leukocytes. Am. J. Vet. Res. 41:1690-1693.

Kaehler, K. L., R. J. F. Markham, C. C. Muscoplat, and D. W. Johnson. 1980b. Evidence of species specificity in the cytocidal effects of Pasteurella haemolytica. Infect. and Immun. 30:615-616.

Kapral, F. A. and M. G. Shayegani. 1959. Intracellular survival of staphylococci. J. Exp. Med. 110:123-138.

Karakawa, W. W. and D. A. Young. 1979. Immunological specificity of heat-stable opsonins in immune and nonimmune sera and their interaction with non-encapsulated and encapsulated strains of Staphylococcus aureus. Infect. and Immun. 25:175-186.

Kato, K. and Y. Bito. 1978. Relationship between bactericidal action of complement and fluidity of cellular membranes. Infect. Immun. 19:12-17.

Keiss, R. E., D. H. Will, and J. R. Collier. 1964. Skin toxicity and hemodynamic properties of endotoxin derived from Pasteurella haemolytica. Am. J. Vet. Res. 25:935-942.

King, B. F., M. L. Biel, and B. J. Wilkinson. 1980. Facile penetration of the Staphylococcus aureus capsule by lysostaphin. Infect. and Immun. 29:892-896.

King, B. F. and B. J. Wilkinson. 1981. Binding of human immunoglobulin G to protein A in encapsulated Staphylococcus aureus. Infect. and Immun. 33:666-672.

Kitt, Sitzungsher, and Geseelsch. 1885. Cited in Gillespie and Timoney, "Hagan and Bruner's Infectious Diseases of Domestic Animals," 7th ed. Ithaca, NY: Cornell University Press, 1981.

Knight, H. D., E. L. Biberstein, and M. Allison. 1969. The role of capsular and somatic antigens in immunization of mice against Pasteurella haemolytica infection. The Cornell Vet. 59:55-64.

Kume, K., A. Sawata, and Y. Nakase. 1978. Haemophilus paragallinarum isolated from chickens affected with coryza. Jap. J. Vet. Sci. 40:65-73.

Leive, L. 1974. The barrier function of the gram-negative envelope. Ann. N. Y. Acad. Sci. 235:109-129.

Lignières, J. 1901. Cited in G. R. Carter, 1968.

Lilli, L. E. 1974. The bovine respiratory disease complex. Can. Vet. J. 15:233-242.

Loos, M., B. Wellek, R. Thesen, and W. Opferkuch. 1978. Antibody-independent interaction of the first component of complement with gram-negative bacteria. Infect. and Immun. 22:5-9.

Maaløe, O. 1948. Pathogenic-apathogenic transformation of Salmonella typhimurium. II. Induced change of resistance to complement. Acta. Pathol. Microbiol. Scand. 25:755-766.

MacDonald, J. T., S. K. Maheswaran, J. Opuda-Asibo, E. L. Townsend, and E. S. Thies. 1983. Susceptibility of Pasteurella haemolytica to the bactericidal effects of serum, nasal secretions, and bronchoalveolar washings from cattle. Vet. Microbiol. 8:585-599.

MacLeod, C. M. and M. R. Krauss. 1950. Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed in vitro. J. Exp. Med. 92:1-9.

Magwood, S. E., D. A. Barnum, and R. G. Thomson. 1969. Nasal bacterial flora of calves in healthy and pneumonia-prone herds. Can. J. Comp. Med. 33:237-243.

Maheswaran, S. K., K. A. Berggren, R. R. Simonson, G. E. Ward, and C. C. Muscoplat. 1980. Kinetics of interaction and fate of Pasteurella haemolytica in bovine alveolar macrophages. Infect. and Immun. 30:254-262.

Maheswaran, S. K. and E. S. Thies. 1979. Influence of encapsulation on phagocytosis of Pasteurella multocida by bovine neutrophils. Infect. and Immun. 26:76-81.

Mandell, G. L. 1975. Effect of temperature on phagocytosis by human polymorphonuclear neutrophils. Infect. Immun. 12:221-223.

Maneval, W. E. 1941. Staining bacteria and yeasts with acid dyes. Stain Technol. 16:13-19.

Maplesden, D. C. and G. R. Carter. 1955. Bovine mastitis caused by Pasteurella haemolytica: A case report. Can. J. Comp. Med. and Vet. Sci. 14:295-296.

Markham, R. J. F., M. L. R. Romnaraine, and C. C. Muscoplat. 1982. Cytotoxic effect of Pasteurella haemolytica on bovine polymorphonuclear leukocytes and impaired production of chemotatic factors by Pasteurella haemolytica-infected alveolar macrophages. Am. J. Vet. Res. 43:285-288.

Markham, R. J. F. and B. N. Wilkie. 1980. Interaction between Pasteurella haemolytica and bovine alveolar macrophages: Cytotoxic effect on macrophages and impaired phagocytosis. Am. J. Vet. Res. 41:18-22.

Martin, S. W., A. H. Meek, D. G. Davis, R. G. Thomson, J. A. Johnson, A. Lopez, L. Stephens, R. A. Curtis, J. F. Prescott, S. Rosendal, M. Savan, A. J. Zubaidy and M. R. Bolton. 1980. Factors associated with mortality in feedlot cattle: The Bruce County beef cattle project. Can. J. Comp. Med. 44:1-10.

Matsuoka, A., T. Folkerts, and C. Gale. 1972. Evaluation in calves of an inactivated bovine rhinotracheitis and Parainfluenza-3 vaccine combined with pasteurella bacterin. J. Am. Vet. Med. Ass. 160: 333-337.

Melching, L. and S. I. Vas. 1971. Effects of serum components on gram-negative bacteria during bactericidal reactions. Infect. Immun. 3:107-115.

Melly, M. A., L. J. Duke, D. F. Liau, and J. H. Hash. 1974. Biological properties of encapsulated Staphylococcus aureus. Infect. Immun. 10:389-397.

Metchnikoff, E. 1887. Cited in W. B. Wood, Jr., 1960.

Mezzatesta, J. R. and R. F. Rest. 1983. Phagocytic killing of Neisseria gonorrhoeae by human monocytes. Infect. and Immun. 42:99-105.

Mian, F. A., T. R. Jarman, and R. C. Righelato. 1978. Biosynthesis of exopolysaccharide by Pseudomonas aeruginosa. J. Bacteriol. 134: 418-422.

Michael, J. G. and W. Braun. 1964. Analysis of sequential stages in serum bactericidal reactions. J. Bacteriol. 87:1067-1072.

Miller, A. W. 1927. Report of committee on miscellaneous transmissable diseases: Hemorrhagic septicemia. J. Am. Vet. Med. Ass. 70: 952-955.

Mishell, B. B. and S. M. Shiig. 1980. Selected Methods in Cellular Immunology. San Francisco: W. H. Freeman and Co., 16-17, 22.

Moore, 1895. Cited in Gillespie and Timoney, 1981.

Morter, R. L., H. E. Amstutz, and R. A. Crandell. 1982. Clinical evaluation of prophylactic regimens for bovine respiratory disease. The Bov. Practitioner 17:56-58.

Mudd, S., M. McCutcheon, and B. Lucké. 1934. Phagocytosis. Physiol. Rev. 14:210-275.

Mueller, R., E. J. Carroll, and L. Panico. 1983. Hemolytic complement titers and complement C_3 levels in endotoxin-induced mastitis. Am. J. Vet. Res. 44:1442-1445.

Mukkur, T. K. S. 1977. Demonstration of cross-protection between Pasteurella multocida type A and Pasteurella haemolytica, serotype 1. Infect. and Immun. 18:583-585.

Mukkur, T. K. S., J. Blunck, and A. Barnes. 1983. Prevention of experimental pneumonia in rams by immunization. Agri-Practice, 4:16-20.

Muschel, L. H., R. H. Chamberlin, and E. Osawa. 1958. Bactericidal activity of normal serum against bacterial cultures. I. Activity against Salmonella typhi strains. Proc. Soc. Expl. Biol. (NY), 97:376-382.

Muschel, L. H. and H. P. Treffers. 1956. Quantitative studies on the bactericidal actions of serum and complement. I. A rapid photometric growth assay for bactericidal activity. J. Immunol. 76:1-10.

Musher, D. M., M. Hague-Park, R. E. Baughn, R. J. Wallace, Jr., and B. Cowley. 1983. Opsonizing and bactericidal effects of normal human serum on nontypable Haemophilus influenzae. Infect. and Immun. 39:297-304.

National Veterinary Services Laboratories. 1979. Diagnostic Bacteriology Laboratory Microtiter Technique Manual. Ames, IA: U. S. Department of Agriculture, Animal and Plant Health Inspection Service.

Newman, P. R., R. E. Corstvet, and R. J. Panciera. 1984. Distribution of Pasteurella haemolytica and Pasteurella multocida in the bovine lung following vaccination and challenge as an indicator of lung resistance. Amer. J. Vet. Res. 43:417-422.

Newsom, I. E. and F. Cross. 1932. Some bipolar organisms found in pneumonia of sheep. J. Am. Vet. Med. Ass. 80:711-719.

Opuda-Asibo, J., E. L. Townsend, S. K. Maheswaran, and J. R. Leininger. 1983. Immunological defense mechanisms against Pasteurella haemolytica in the bovine lung. Abst. N. A. Symp. Bov. Res. Dis. Amarillo, TX.

Osawa, E. and L. H. Muschel. 1964. Studies relating to the serum resistance of certain gram-negative bacteria. J. Exp. Med. 119:41-51.

Otulakowski, G. L., P. E. Shewen, A. E. Udoh, A. Mellors, and B. N. Wilkie. 1983. Proteolysis of sialoglycoprotein by Pasteurella haemolytica cytotoxic culture supernatant. Infect. and Immun. 42:64-70.

Palotay, J. L. and N. R. Christensen. 1959. Bovine respiratory infections. I. Psittacosis-lymphogranuloma venereum group of viruses as etiological agents. J. Am. Vet. Med. Ass. 134:222-230.

Palotay, J. L., S. Young, S. A. Lovelace, and J. H. Newhall. 1963. Bovine respiratory infections. II. Field trial using bacterial vaccine products as a means of prophylaxis. Am. J. Vet. Res. 24:1137-1142.

Panciera, R. J. and R. E. Corstvet. 1984. A model for Pasteurella haemolytica and Pasteurella multocida-induced pneumonia in cattle. Am. J. Vet. Res. in press.

Panciera, R. J., R. E. Corstvet, A. W. Confer, and C. N. Gresham. 1984. Bovine pneumonic pasteurellosis: Effect of vaccination with live Pasteurella species. Am. J. Vet. Res. in press.

Pass, D. A. and R. G. Thomson. 1971. Wide distribution of Pasteurella haemolytica type 1 over the nasal mucosa of cattle. Can. J. Comp. Med. 35:181-186.

Pasteur, L. 1880. Cited in Gillespie and Timoney, 1981.

Patriarca, P., S. Beckerdite, and P. Elsbach. 1972. Phospholipases and phospholipid turnover in Escherichia coli spheroplasts. Biochem. Biophys. Acta. 260:593-600.

Peterson, P. K., Y. Kim, B. J. Wilkinson, D. Schmeling, A. F. Michael, and P. G. Quie. 1978. Dicotomy between opsonization and serum complement activation by encapsulated Staphylococci. Infect. and Immun. 20:770-775.

Quie, P. G., R. P. Messner, and R. C. Williams, Jr. 1968. Phagocytosis in subacute bacterial endocarditis: Localization of the primary opsonic site to Fc fragment. J. Exp. Med. 128:553-570.

Rehmtulla, A. J. and R. G. Thomson. 1981. A review of the lesions in shipping fever of cattle. Can. Vet. J. 22:1-8.

Reynolds, B. L. and H. Pruul. 1971. Sensitization of complement-resistant smooth gram-negative bacterial strains. Infect. and Immun. 3:365-372.

Rhodes, M. W. and H. S. Hsu. 1974. Effect of kanamycin on the fate of Salmonella enteritidis with cultured macrophages of guinea pigs. J. Reticuloendo. Soc. 15:1-12.

Roberts, N. J. and R. T. Steigbigel. 1977. Hyperthermia and human leukocyte functions: Effects on response of lymphocytes to mitogen and antigen and bactericidal capacity of monocytes and and neutrophils. Infect. and Immun. 18:673-679.

Rossi, C. R. and G. K. Kiesel. 1974. Serological evidence for the association of bovine respiratory syncytial virus with respiratory tract disease in Alabama cattle. Infect. and Immun. 10:293-298.

Roth, J. A., M. L. Kaeberle, and R. W. Griffith. 1981. Effects of bovine viral diarrhea virus infection on bovine polymorphonuclear leukocyte function. Am. J. Vet. Res. 42:244-250.

Rothbard, S. 1948. Protective effect of hyaluronidase and type-specific anti-M serum on experimental group A streptococcus infections in mice. J. Exp. Med. 88:325-341.

Rowley, D. 1968. Sensitivity of rough gram-negative bacteria to the bactericidal action of serum. J. Bacteriol. 95:1647-1650.

Rowley, D. 1971. Endotoxins and bacterial virulence. J. Infect. Dis. 123:317-327.

Rowley, D. 1973. Antibacterial action of antibody and complement. J. Inf. Dis. 128:S170-S175.

Rush, H. G., J. C. Glorioso, C. A. Darif, and L. C. Olson. 1981. Resistance of Pasteurella multocida to rabbit neutrophil phagocytosis and killing. Am. J. Vet. Res. 42:1760-1768.

Sawyer, W. D. 1969. Interaction of influenza virus with leukocytes and its effect on phagocytosis. J. Infect. Dis. 119:541-556.

Schmid, E. N., B. Menge, and K. G. Lickfeld. 1981. Capsule fine structure in thin slices of Klebsiella pneumonia Biovar d (Richard). J. Ultrastruct. Res. 75:41-49.

Schwab, J. H. 1975. Suppression of the immune response by microorganisms. Bacteriol. Res. 39:121-143.

Scott, J. P. and H. Farley. 1932. Preliminary bacteriological report on shipping fever. J. Am. Vet. Med. Ass. 80:173-186.

Shewen, P. E. and B. N. Wilkie. 1982. Cytotoxin of Pasteurella haemolytica acting on bovine leukocytes. Infect. and Immun. 35: 91-94.

Shewen, P. E. and B. N. Wilkie. 1983a. Pasteurella haemolytica cytotoxin: Production by recognized serotypes and neutralization by type-specific rabbit antisera. Am. J. Vet. Res. 44:715-719.

Shewen, P. E. and B. N. Wilkie. 1983b. Pasteurella haemolytica cytotoxin neutralizing activity in sera from Ontario beef cattle. Can. J. Comp. Med. 47:497-498.

Smith, G. R. 1959. Isolation of two types of Pasteurella haemolytica from sheep. Nature 183:1132-1133.

Smith, G. R. 1960. Virulence and toxicity of Pasteurella haemolytica in the experimental production of ovine septicemia. J. Comp. Path. and Ther. 70:429-436.

Smith, H. 1977. Microbial surfaces in relation to pathogenicity. Bacteriol. Rev. 41:475-500.

Smith, J. E. 1974. Genus Pasteurella. Trevisan, 1887, p. 372. In R. E. Buchanan and N. E. Gibbons (eds.). Bergey's Manual of Determinative Bacteriology, 8th ed. Baltimore: The Williams and Wilkins Co.

Stinson, M. W. and C. J. van Oss. 1971. Immunoglobulins as aspecific opsonins. II. The influence of specific and aspecific immunoglobulins on the in vitro phagocytosis of noncapsulated, capsulated, and decapsulated bacteria by human neutrophils. J. Reticuloend. Soc. 9:503-512.

Stossel, T. P. 1973. Quantitative studies of phagocytosis: Kinetic effects of cations and of heat-liable opsonin. J. Cell. Biol. 58:346-356.

Stossel, T. P. 1975. Phagocytosis: Recognition and ingestion. Seminars in Hematol. 12:83-116.

Sutherland, I. W. 1972. Bacterial exopolysaccharides. Adv. Microb. Physiol. 8:143-213.

Tadayon, R. A. and L. H. Lauerman. 1981. The capacity of various fractions of Pasteurella haemolytica to stimulate protective immunity in mice and hampsters. Vet. Microbiol. 6:85-93.

Tajima, M., T. Yagihashi, and Y. Miki. 1982. Capsular material of Mycoplasma gallisepticum and its possible relevance to the pathogenic process. Infect. and Immun. 36:830-833.

Taylor, P. W. 1975. Genetical studies of serum resistance in Escherichia coli. J. Gen. Microbiol. 89:57-66.

Taylor, P. W. 1983. Bactericidal and bacteriolytic activity of serum against gram negative bacteria. Microbiol. Rev. 47:46-83.

Taylor, P. W. and H. P. Kroll. 1983. Killing of an encapsulated strain of Escherichia coli by human serum. Infect. and Immun. 39:122-131.

Thomson, R. G. 1980. A perspective on respiratory disease in feedlot cattle. Can. Vet. J. 21:181-185.

Thomson, R. G. 1981. The pathogenesis of shipping fever in cattle. Bovine Proceedings 13:103-112.

Trevisan. 1887. Cited in Gillespie and Timoney, 1981.

Troy, F. A. 1973. Chemistry and biosynthesis of the poly (γ-D-glutamyl) capsule in Bacillus licheniformis. J. Biol. Chem. 248:305-315.

Troy, F. A. 1979. The chemistry and biosynthesis of selected bacterial capsular polymers. Ann. Rev. Microbiol. 33:519-560.

Turner, W. G. 1916. Shipping fever from the Army standpoint. J. Am. Vet. Med. Ass. 50:594-600.

Tweed, W. and J. W. Eddington. 1930. Pneumonia in the bovine due to Pasteurella boviseptica. J. Comp. Path. 43:234-252.

van Furth, R. and T. L. van Zwet. 1973. In vitro determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. pp. 36.1-36.24 in Handbook of Experimental Immunology, D. W. Weir and M. D. Edin (eds.). Oxford, England: Blackwell Scientific Publications.

van Oss, C. J. 1978. Phagocytosis as a surface phenomenon. Ann. Rev. Microbiol. 32:19-39.

Verbrugh, H. I., W. C. van Dijk, M. E. van Erne, R. Peters, P. K. Peterson, and J. Verhoef. 1979. Quantitation of the third component of human complement attached to the surface of opsonized bacteria: Opsonin-deficient sera and phagocytosis-resistant strains. Infect. and Immun. 26:808-812.

Verhoef, J., P. K. Peterson, and P. G. Quie. 1977. Kinetics of Staphylococcal opsonization, attachment, ingestion and killing by human polymorphonuclear leukocytes: A quantitative assay using [^{3}H] thymidime labelled bacteria. J. Immunol. Meth. 14:303-311.

Walker, R. D., R. E. Corstvet, and R. J. Panciera. 1980. Study of bovine pulmonary response to Pasteurella haemolytica: Pulmonary macrophage response. Am. J. Vet. Res. 41:1008-1014.

Ward, H. K. and J. F. Enders. 1933. An analysis of the opsonic and tropic action of normal and immune sera based on experiments with the pneumococcus. J. Exp. Med. 57:527-547.

Wells, P. W., N. J. L. Gilmour, C. Burrells, and D. A. Thompson. 1979. A serological comparison of Pasteurella haemolytica vaccines containing different adjuvents. Res. in Vet. Sci. 27:248-250.

Wessman, G. E. 1964. Interrelationships of smooth and nonsmooth variants in the dissociation of Pasteurella haemolytica. J. of Bacteriol. 88:356-360.

Whitnack, E., A. L. Bisno, and E. H. Beachey. 1981. Hyaluronate capsule prevents attachment of Group A streptococci to mouse peritoneal macrophages. Infect. and Immun. 31:985-991.

Wilkie, B. N. 1981. Principles of biological control of shipping fever. The Bov. Proceedings 13:113-114.

Wilkie, B. N. 1982. Respiratory tract immune response to microbial pathogens. J. Am. Vet. Med. Ass. 181:1074-1079.

Wilkie, B. N. and R. J. F. Markham. 1979. Sequential titration of bovine lung and serum antibodies after parenteral or pulmonary inoculation with Pasteurella haemolytica. Am. J. Vet. Res. 40: 1690-1693.

Wilkie, B. N., R. J. F. Markham, and P. E. Shewen. 1980. Response of calves to lung challenge exposure with Pasteurella haemolytica after parenteral or pulmonary immunization. Am. J. Vet. Res. 41:1773-1778.

Wilkie, B. N. and A. M. Norris. 1975. The effect of route of immunization on the lapine immune response to killed Pasteurella haemolytica and the influence of aerosol challenge with the live organism. Can. J. Comp. Med. 40:346-349.

Wilkinson, B. J., P. K. Peterson, and P. G. Quie. 1979a. Cryptic peptidoglycan and the antiphagocytic effect of the Staphylococcus aureus capsule: Model for the antiphagocytic effect of bacterial cell surface polymers. Infect. and Immun. 23:502-508.

Wilkinson, B. J., S. P. Sisson, Y. Kim, and P. K. Peterson. 1979b. Localization of the third component of complement on the cell wall of encapsulated Staphylococcus aureus M: Implications for the mechanism of resistance to phagocytosis. Infec. and Immun. 26: 1159-1163.

Wilkinson, J. F. 1958. The extracellular polysaccharides of bacteria. Bacteriol. Rev. 22:46-73.

Williams, A. G. and J. W. T. Wimpenny. 1977. Exopolysaccharide production by Pseudomonas NCIB11264 grown in batch culture. J. Gen. Microbiol. 102:13-21.

Williams, P., P. A. Lambert, M. W. Brown, and R. J. Jones. 1983. The role of the O and K antigens in determining the resistance of Klebsiella aerogenes to serum killing and phagocytosis. J. Gen. Microbiol. 129:2181-2191.

Winter, A. J. 1979. Mechanisms of immunity in bacterial infections. Adv. Vet. Sci. Comp. Med. 23:53-69.

Wood, W. B., Jr. 1960. Phagocytosis, with particular reference to encapsulated bacteria. Bacteriol. Rev. 24:41-49.

Wood, W. B. and M. R. Smith. 1949. The inhibition of surface phagocytosis by the capsular "slime layer" of pneumococcus type III. J. Exp. Med. 90:85-96.

Woods, G. T., M. E. Mansfield, and G. Marquis. 1974. A four-year clinical and serologic study of the use of inactivated para-influenzae-3 virus vaccines and Pasteurella sp. bacterins in beef calves. Vet. Med./Sm. An. Clin. 69:474-478.

Wright, A. E. and S. R. Douglas. 1903. Cited in W. B. Woods, Jr., 1960.

Yates, W. D. G., P. H. G. Stockdale, L. A. Babiuk, and R. J. Smith. 1983. Prevention of experimental bovine pneumonic Pasteurellosis with an extract of Pasteurella haemolytica. Can. J. Comp. Med. 47:250-256.

VITA 2

Martha Joy Gentry

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE ROLE OF CAPSULAR MATERIAL OF PASTEURELLA HAEMOLYTICA, TYPE 1, IN BOVINE PNEUMONIC PASTEURELLOSIS

Major Field: Veterinary Parasitology and Public Health

Biographical:

Personal Data: Born in Independence, Missouri, October 1, 1947, the daughter of Mr. and Mrs. Lloyd M. Seitz.

Education: Graduated from Junction City High School, Junction City, Kansas, in May, 1965; received Bachelor of Science degree in Bacteriology from Kansas State University in 1969; received Master of Arts degree in Microbiology from Indiana University in 1971; completed the requirements for Doctor of Philosophy degree at Oklahoma State University in May, 1984.

Professional Experience: Laboratory Technician, Dykstra Veterinary Hospital, Kansas State University, 1968-1969; Graduate Teaching Assistant, Department of Microbiology, Indiana University, 1970-1971; Microbiologist, Bloomington Hospital, Bloomington, Indiana, 1971-1973; Assistant Instructor, College of Veterinary Medicine, Kansas State University, 1973-1974 and 1977-1978; Research Assistant, Department of Microbiology, Kansas State University, 1975-1976; Student Paraprofessional, Department of Veterinary Parasitology, Microbiology, and Public Health, Oklahoma State University, 1980-1984.