THE DISTRIBUTION OF *PASTEURELLA HAEMOLYTICA* AND *PASTEURELLA MULTOCIDA* IN THE LUNGS OF CATTLE AFTER VACCINATION AND CHALLENGE AS AN INDICATOR OF LUNG RESISTANCE

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CHAPTER I

INTRODUCTION

Shipping fever or acute pneumonic pasteurellosis, as a part of the the bovine respiratory disease complex, remains a major cause of morbidity and mortality in feedlot cattle and results in major economic loss to the cattle industry. This disease appears to be of multifactorial etiology with bacterial agents responsible for the severe pneumonia typical of the disease. The bacteria most frequently isolated from clinical cases of shipping fever are *Pasteurella haemolytica* and *Pasteurella multocida*. The disease is characterized clinically by anorexia, fever, depression, nasal discharge and referred bronchial and pleuritic sounds in the anteroventral parts of the lungs, pathologically by acute inflammation of the airways, fibrinous alveolitis, intravascular thrombosis, lobular necrosis, and fibrinous pleuritis, and epizootiologically by the development of the disease within 45 days after shipment (1). Shipping fever may result in death losses which may approach three to five per cent (2). In one feed lot, respiratory disease of the shipping fever type was responsible for about 75 per cent of all clinical

diagnoses and 64 per cent of all necropsy diagnoses (1). Shipping fever pneumonia typically appears after the sequential interaction of stressors, viruses and virulent bacteria (3).

Preceeding viral infections and other factors are believed to impair pulmonary bacterial clearance allowing the pasteurellae to produce disease (4, 5, 6). The role of infectious bovine rhinotracheitis (IBR) virus as an important agent in bovine respiratory disease is well established, being capable of producing disease as the sole etiological agent (7, 8) and implicated as apredisposing agent in the shipping fever complex (9, 10).

This study was designed to gain information on the relationship between _P. haemolytica_ and _P. multocida_ and the host pulmonary cells in cattle which had been experimentally vaccinated and subsequently challenged by direct intrapulmonic injection with the homologous strain of either _P. haemolytica_ or _P. multocida_ used in the vaccination. The objectives were 1) to determine whether the fluorescent antibody technique could be used to demonstrate bacteria in the lungs of calves, 2) to determine the relative numbers, distribution and integrity of the bacteria in the lungs, 3) to determine the effect of an experimental IBR virus infection on the relative numbers, distribution and integrity of bacteria in the lungs of a susceptible calf, and 4) to determine if electron microscopy and the immunoperoxidase pro-

cedure could be used to demonstrate bacteria in the lungs of calves. Results from these studies were analyzed to see if the relative numbers, distribution and integrity of bacteria in the lungs of calves could be correlated with lung resistance as determined by other parameters.

CHAPTER II

REVIEW OF THE LITERATURE

Microorganisms in the genus *Pasteurella* have been associated with disease almost from the time of the germ theory of disease and the discovery of bacteria, and diseases now known to be caused by *Pasteurella* species were reported long before the isolation of the etiological agent. Dadd (11) discussed the the clinical signs and treatment of respiratory diseases called catarrh or enzootic catarrh in cattle and made reference to the fact that the disease occurred when animals were moved from a warm to a colder region and suggested this was due to an hereditary predisposition. Bollinger (12), in 1878, made what was probably the first significant report of what was to be labelled pasteurellosis in an outbreak of fatal disease among wild animals and cattle. An agent now identified as *Pasteurella multocida* was known as the etiological agent of fowl cholera in 1880 (13). Poels (12) was the first to describe this organism involved in calf pleuropneumonia. In 1895, Moore (14) reported finding *Pasteurella* present on the mucous membranes of normal cattle.

The genus name *Pasteurella* was suggested in 1887

in honor of Louis Pasteur (15). Due to the broad host range of the organism many synonyms for the same organism were used depending on the species of host harboring the infection, with *P. boviseptica* used for the strains of the organism isolated from cattle. However, as it became apparent that all the organisms produced similar diseases and were morphologically, biochemically and serologically similar, an attempt was made to include all the strains under one name. The name *Pasteurella multocida* was proposed in 1939 for all isolates regardless of the source of the organism (16).

Pasteurella haemolytica was apparently first isolated from cases of pneumonia in calves in 1921 but was considered to be a variant of *P. multocida* with the ability to hemolyze red blood cells (17). The organism was recovered from an outbreak of infectious pneumonia in dairy cattle in England in 1930 (18). The disease was described as being associated with newly purchased animals shortly after arrival at their destination. The organism was thought to be *P. boviseptica* (*P. multocida*) because of agglutination and absorption of agglutinins but the organism was hemolytic and grew on MacConkey agar, both characteristics of *P. haemolytica*. The isolate was also used to inoculate rabbits by intravenous, subcutaneous and intraperitoneal routes and failed to produce disease. These pathological findings were also characteristic of *P. haemolytica* and not of *P.*

multocida. The organism from the lungs of one of the calves was isolated in pure culture, grown in agar culture, suspended in saline and injected intravenously into a healthy calf. The animal died 36 hours later with a pneumonia typical of that seen in the calf from which the organism was isolated. The organism was first isolated in the United States from sheep in 1932 by Newsom and Cross (19) who named the organism *P. haemolytica*. They suggested the species should include all isolates that were hemolytic, did not produce indole, were non-virulent for rabbits and produced acid in unheated dextrin, inositol and maltose.

The involvement of both *P. multocida* (*P. boviseptica*) and *P. haemolytica* in shipping fever has been described by many workers. The disease was called "transit fever", "stockyards pneumonia" and "haemorrhagic septicemia". An early report described the disease in calves after shipment (20). Other reports gave decriptions of the disease manifested by bronchopneumonia from which *P. multocida* or *P. haemolytica* were isolated (18, 21, 22, 23, 24, 25).

Investigations in the early 1950's by Carter (26, 27, 28) indicated the importance of *P. haemolytica* in shipping fever of calves. In these studies *P. haemolytica* was the most commonly isolated organism from cases of shipping fever. The importance of *P. multocida* in shipping fever was indicated by the frequency of

isolation of the organism from lesions in cases of shipping fever (26,29,30,31); and by the fact that isolations from the nasopharynx were much higher in cattle with shipping fever than normal cattle (32, 33). Hoerlein et al. (33) isolated Pasteurella species from the nasopharynx of 59.6 per cent of calves with shipping fever but from only three per cent of normal calves.

Despite these findings the role of P. multocida and P. haemolytica in shipping fever has not always been certain. Many authors were unsure of the actual role of these organisms because of the difficulty in reproducing the disease experimentally with these agents. The organisms were not always isolated from cases of shipping fever and were beleived by some to be secondary invaders of compromised lung after the pneumonia of shipping fever was established and not the primary agent in shipping fever (34, 35). Attempts were made to experimentally reproduce the disease by exposing cattle to various types of presumably infectious material. Gale and Smith (36) exposed calves to cultures of P. multocida and P. haemolytica subcutaneously and/or intratracheally . Signs of respiratory disease were not produced in any of the calves inoculated by the various routes; none of the calves had a febrile response and none had an altered blood picture or any evidence of pneumonia at necropsy. Gale, King and Sanger (37) attempted to transmit the shipping fever pneumonia from

affected animals to apparently healthy ones. The authors took nasal washings, lung suspensions and defibrinated blood from acutely ill animals and inoculated the specimens into healthy calves by intravenous, intratracheal, intranasal or subcutaneous routes. A pneumonia typical of the acute clinical disease affecting calves from which material was taken usually did not develop in these calves, although some calves did have a febrile response and signs of respiratory illness. Only two of thirty-five calves developed pneumonic lesions from the inoculation. These lesions were similar to those seen in natural cases but were not as severe.

Pasteurella haemolytica and *P. multocida* were also frequently isolated from normal healthy cattle (32, 38, 39). Magwood et al. (39) found *P. haemolytica* and *P. multocida* from nasal swabs with equal frequency from cattle which were or which would become ill as from normal calves which did not become ill. They concluded there was no characteristic nasal flora which was an indicator of future disease and that *Pasteurella* species were capable of dominating the nasal flora on a transient basis without causing disease.

The role of *P. haemolytica* and *P. multocida* in the bovine respiratory disease complex has been the subject of much study. The sum total of epizootiological and clinical, as well as experimental, evidence available has now established *P. haemolytica* and *P. multocida* as

essential components in the etiology of shipping fever (40, 41).

The role of viral infections as predisposing agents in shipping fever has been known for some time, and viruses are beleived to be an intrinsic part of the respiratory disease complex (42, 43, 44, 45). Viral agents were often isolated from cases of shipping fever along with the Pasteurella or in some cases when Pasteurella were not isolated. At one time a virus was thought to be the primary etiological agent in shipping fever. Ryff and Glenn (46) isolated a viral agent, which they believed to be the primary etiological agent, from clinical shipping fever along with P. multocida. The virus was not characterized due to the inability to maintain the virus in embryonated eggs. The first virus to be characterized from shipping fever was a hemagglutinating virus of the Myxovirus group (47). The virus isolated was later found to be identical with the para-influenza$_3$ virus and this virus received much of the early attention of investigators.

The IBR virus was first recognized as an entity in beef cattle in Colorado in 1950 and was first reported as a respiratory pathogen in 1955 (48). The disease was reported to have occurred sporadically in 1951 and epizootiologically in 1952. The disease spread from one of mature feedlot cattle to a disease also affecting young cattle and dairy cattle. The disease was reported to

have had the highest incidence in beef calves in feedlots seven to ten days after their apparent recovery from shipping fever. At a meeting of the United States Livestock Sanitary Association in November, 1955 it was generally agreed to call this disease infectious bovine rhinotracheitis (IBR) (49).

The virus was first isolated in 1956 when grown on bovine embryonic kidney cells (49, 50). Isolations were made from nasal washings and tissue from calves in the early acute phase of illness. Two to three days after inoculation with infectious material a cytopathogenic effect was noted on the tissue cultured cells. The cells became more granular and refractile in appearance and rounded in shape. Inoculation of the infected tissue culture fluid intranasally into cattle resulted in reproduction of the clinical signs observed in natural cases of the disease. The virus was included in the herpes group based on its size, type of intranuclear inclusion bodies, cytopathic effect in tissue culture and other physical and chemical properties (51). Because of this inclusion in the herpes group the virus is often called bovine herpesvirus 1 (52). Following the initial reports, IBR was diagnosed with increasing frequency and it soon came to be recognized as a cattle disease of major economic importance (53). Soon after the isolation of the IBR virus work was done on determining whether the virus potentiated the action of P.

haemolytica on cattle (54). The results indicated that *P. haemolytica* or the IBR virus alone could cause clinical signs of respiratory disease but signs were most severe when both agents were present. The effect was not great, being mostly an increased febrile response.

The studies of IBR viral infection indicate that the virus has an effect on the tracheal epithelium ranging from partial denuding to complete sloughing of the columnar epithelium (55). Viral pathogens are also capable of destroying the so-called "mucus escalator" of the respiratory tract by infecting the ciliated and mucus secreting cells (56). This effect could account for a portion of the predisposing effect of the virus to bacterial infection. It has become recognized that *P. haemolytica* and *P. multocida* and the various viral agents are all involved in bovine respiratory disease, and that any of the agents alone may cause respiratory disease but quite often they act together either by addition or synergism (57).

The combination of IBR virus and *P. haemolytica* was found to produce clinical pneumonia when the virus was given as an aerosol three to five days prior to an aerosol of *P. haemolytica* (9). All calves exposed to both the viral and bacterial aerosols developed clinical signs of elevated temperatures, muco-purulent nasal discharge and, in a few instances, death of the animal. Pneumonia similar to that of natural shipping fever was

seen upon necropsy of those animals that died. The exposure of calves to aerosols of *P. haemolytica* only failed to produce any respiratory disease and an aerosol of *P. haemolytica* failed to produce signs of respiratory disease if given less than three days after the IBR aerosol. Success was reported in preventing pneumonia by vaccinating the calves with IBR vaccine prior to the IBR and *Pasteurella* aerosol (10).

CHAPTER III

MATERIALS AND METHODS

Microorganisms

Pasteurella multocida, serotype three, (58) and *P. haemolytica*, serotype one, (59) were originally isolated from the tracheas of feedlot cattle using tracheal swabs. The virulence of each was maintained by occasional passage through calves by intrapulmonic inoculation and reisolation of the organims from the resulting lung lesion. The isolate was passed twice on solid medium incubated at 37° C in a candle jar, suspended in sterile skim milk, lyophilized and stored at -20° C. The bacterial suspension utilized for vaccination and challenge was prepared as previously described (60). The concentration of bacteria used in the experimental procedures was approximately 1.0×10^{9} colony forming units/ml (CFU/ml), with a range of 9.5×10^{8} to 1.7×10^{9} CFU/ml as measured by a modified plate count method (61). Impinger samples were taken during the aerosol vaccination using 20 ml of brain heart infusion broth (Difco Laboratories) and were counted with the spot plate count.

The medium used for growth and maintenance of the *Pasteurella* species was brain heart infusion (Difco Laboratories) agar with five per cent citrated bovine blood, one per cent horse serum (Pel-Freez), and one per cent filtered yeast hydrolysate (ICN Pharmaceuticals). The bacteria were suspended in 0.85% NaCl 0.01M phosphate buffer pH 7.2 (PBS) for the vaccination and challenge procedures. Incubation of the *Pasteurella* species was always at $37^{o}C$ in candle jars.

The infectious bovine rhinotracheitis (IBR) virus was originally isolated from the serum of a naturally infected calf by the Oklahoma Animal Disease Diagnostic Laboratory. The virus was passed three times *in toto* on bovine embryonic lung cells grown in Dulbeccos modified essential medium (International Scientific Instruments) with 10 per cent bovine fetal serum (Microbiological Associates) and 40 micrograms gentamicin/ml (Schering Corporation). The virus was frozen at -70° C until used to inoculate the calves. At that time the virus was determined to have a titer of 1.0×10^{6} tissue culture infective dose/ml (TCID/ml) as determined by cytopathic effect and the direct fluorescent antibody technique (conjugated antiserum from Microbiological Associates).

Production of Antiserum

Antiserum to the *Pasteurella* species for use in the fluorescent antibody technique was prepared in eight

week old chickens (Arbor Acres Farms). A lyophilized culture of P. haemolytica was rehydrated with sterile water, passed once on solid medium for 20-22 hours, suspended in PBS, and administered as a slightly turbid suspension (McFarland standard three) of the live culture, one ml intravenously four times at weekly intervals. A lyophilized culture of P. multocida was rehydrated with sterile water passed once on the solid medium for 20-22 hours and harvested into a .85% NaCl .3% formalin solution and held at 4° C for 24 hours. This was administered as a slightly turbid suspension (McFarland standard three) of the culture mixed in equal parts with Freunds incomplete adjuvant (Difco Laboratories). One ml of the mixture was injected subcutaneously in the midportion of the neck. The killed cell suspension (McFarland standard three) without adjuvant was also given as a one ml intravenous dose the same day as the suspension with adjuvant and twice more at weekly intervals. Ten days after administration of the final dose of formalin killed cells, one ml of the live culture, passed twice on solid medium after rehydration of the lyophilized culture (McFarland standard three), was given intravenously. Blood was collected from all chickens 14 days after the final injection. The serum was harvested, frozen at -20° C and aliquots were conjugated as needed with fluorescein isothiocyanate isomer 1 (BBL Laboratories) according to

the method of Corstvet and Sadler (62).

Antiserum for the immunoperoxidase procedure was prepared in rabbits. A lyophilized culture of P. haemolytica was rehydrated and passed twice on solid medium and harvested into PBS. This suspension was adjusted to a McFarland standard three density and given intravenously in the ear vein four times at weekly intervals. Serum was harvested from the rabbits 14 days after the final injection of P. haemolytica and frozen in aliquots until used.

Experimental Animals

Forty-seven Hereford, Angus or Hereford-Angus crossbred male and female calves six to eight months old weighting 350-500 pounds were used (Table I, Appendix). Sixteen were used in the P. haemolytica experiment, 28 were used in the P. multocida experiment and three were used in the IBR-P. haemolytica experiment. Calves used in the P. haemolytica experiment were divided into four groups of four calves each. One group was vaccinated by 15 minute exposure to an aerosol of P. haemolytica, one group was sham vaccinated by 15 minute exposure to an aerosol of PBS, one group was vaccinated by a subcutaneous injection of five ml of a P. haemolytica suspension and the fourth group received a subcutaneous injection of five ml of PBS. These procedures were repeated seven days later.

The calves in the *P. multocida* experiment were divided into eight groups. Six calves were vaccinated by 15 minute exposure to an aerosol of *P. multocida*, two calves by eight minute exposure to an aerosol of *P. multocida*, two calves by a three minute exposure to an aerosol of *P. multocida* and five calves received five ml of *P. multocida* suspension subcutaneously. Six calves were sham vaccinated by 15 minute exposure to an aerosol of PBS, one calf by eight minute exposure to an aerosol of PBS, one calf by three minute exposure to an aerosol of PBS and five calves were sham vaccinated subcutaneously with five ml of PBS. The procedures were repeated seven days later.

Three nonvaccinated calves were used in the IBR-*P. haemolytica* experiment. The calves in this experiment were challenged with *P. haemolytica* five days after intratracheal exposure to IBR virus.

Calves in all experiments were monitored prior to and during the experiment by taking nasal and tracheal swabs for culture and drawing blood samples to determine serum antibody titers to IBR virus, bovine viral diarrhea virus, and $parainfluenza_3$ virus.

Aerosol Procedure

The organism to be administered in the aerosol procedure was suspended in PBS at a concentration of $1.0x10^9$ CFU/ml (range $1.0x10^9$ $-1.3x10^9$ CFU/ml). The

aerosol was created with a DeVilbis model 65 ultrasonic nebulizer (DeVilbis Company) and delivered via a flexible one inch diameter, 24 inch long plastic hose to a ten gallon plastic bag (Glad Kitchen Garbage Bag, Union Carbide). The plastic bag was attached to the calves muzzle over a one inch thick foam rubber band fastened in place by elastic tape midway between the nostrils and the eyes. Continuous flow of the aerosol suspension through the bag was accomplished with a rheostat controlled exhaust fan. The rheostat was adjusted to maintain proper inflation of the bag. The exhaust system included a fiberglass prefilter and two biological HEPA filters (Cambridge Filter Company). The relative concentration of bacteria contained in the aerosol suspension was monitored by obtaining an impinger sample using glass impingers (Ace Glass Incorporated).

Challenge Procedure

Challenge was accomplished by transthoracic injection of the bacterial suspension directly into the diaphragmatic lobe of each lung. A site was selected at the eighth intercostal space four to five inches below the vertebral arch on the left and right sides. The site was then washed with soap and water and shaved. Following infiltration of the inoculation site with two per cent lidocaine hydrochloride a 14 gauge one and one

half inch needle was introduced through the skin and served as a canula. A 19 gauge four inch needle was passed through the canula and directed into the diaphragmatic lobe of the lung where five ml of the culture was deposited.

All calves were challenged with the homologous Pasteurella species two weeks after the second vaccination. The three calves used in the IBR-P. haemolytica experiment were challenged with P. haemolytica five days after intratracheal injection of IBR virus (1.5×10^6 TCID contained in 1.5 ml). Calves which died following challenge were necroposied and samples were taken for the fluorescent antibody staining procedure from the lungs and other body tissues. All calves that survived challenge were killed four days post challenge, except in the IBR-P. haemolytica experiment where one calf was killed four days post challenge and two calves were killed five days post challenge.

Fluorescent Antibody Technique (FAB)

Frozen sections six to eight micrometers thick were cut from the center of the lesion produced by the intrapulmonic inoculation from left and right lungs. The sections were dried at 37° C for one hour, fixed in acetone for 10 minutes at room temperature and stored at -20° C until stained. The appropriate dilution of conjugated antiserum was determined by using serial

dilutions of the antiserum applied to smears of the bacteria. The conjugated antiserum was diluted to the proper concentration in 20 per cent bovine tissue homogenate to reduce the nonspecific fluorescence. This homogenate was comprised of lung, liver, heart and kidney obtained from a clinically normal animal and homogenized in PBS in a blender. Tissue sections were evenly flooded with conjugate, incubated for 25 minutes in a moist chamber at 37° C, washed in two changes of PBS to remove excess conjugate, rinsed in distilled water, air dried and mounted in 10 per cent glycerol: 90 per cent PBS. Sections were examined using a Zeiss fluorescence microscope with epiillumination.

Electron Microscopy

Tissue samples for electron microscopy were taken from the lungs within 20 minutes of the time of death of the animal. Samples were taken from the center of the challenge produced lesion, from the edge of the lesion and from normal lung tissue approximately two to four centimeters away from the margin of the lesion. The tissue was minced with scissors and a razor blade and placed in two per cent glutaraldehyde in .27 Molar cacodylate buffer, pH 7.2, for two hours. The samples were washed three times in cacodylate buffer for 20 minutes each wash. Samples were then post fixed in two per cent OsO_4 mixed in equal parts cacodylate buffer and left at

room temperture for two hours. Samples were then washed with three 20 minute water washes and enbloc stained with one-half per cent aqueous uranyl acetate over night at 50°C. The following morning samples were washed with one 20 minute water wash and put through a graded alcohol series, then placed in three changes of propylene oxide for 20 minutes each. Five pieces of tissue from each sample were then transferred to a solution of equal parts propylene oxide and embedding resin (Polybed, Polysciences) for 12 hours, the caps on the vials were removed and samples left for 12 additional hours. The tissues were then transferred to the embedding resin and placed in a vacuum oven at 60°C for 48 hours. The blocks were trimmed and one micron sections were cut from the blocks and stained with Mallory's stain. The sections were examined using a light microscope and an area was selected for thin sectioning. Thin sections were cut, mounted on grids and stained with uranyl acetate and lead citrate. A Philips EM 200 transmission electron microscope was used to examine the sections and electron micrographs taken as required.

Immunoperoxidase Procedure

Tissue sections for the immunoperoxidase procedure were duplicates of those used in the FAB. The samples were flooded with various dilutions of antiserum against *P. haemolytica* prepared in rabbits, and incubated at

37°C for 25 minutes in a moist chamber. The slides were removed and washed in three changes of PBS and rinsed in distilled water. Various dilutions of horseradish peroxidase conjugated goat anti-rabbit antiglobulin (Cappel Laboratories) were flooded onto the tissue sections which were again incubated for 25 minutes at 37°C in a moist chamber. Slides were removed and washed in three changes of PBS and rinsed in distilled water.

The peroxidase method used was that described by Mesulam (63). The sections were placed in a pre-reaction soak for 20 minutes at room temperature. The pre-reaction solution was prepared by mixing two separate solutions, A and B. Solution A contained 92.5 ml distilled water, 100 mg sodium nitroferricyanide, and five ml of 0.2 Molar acetate buffer at pH 3.3 (each 100 ml of this buffer contained 20 ml of 1.0 Molar sodium acetate, 19 ml of 1.0 Molar HCl and distilled water to bring the volume to 100 ml). Solution B contained 5 mg of 3,3',5,5' tetramethyl benzidine (TMB, Sigma) in 2.5 ml absolute ethanol and this was heated up to 40°C in order to dissolve the TMB. Solution A was added to solution B in order to obtain 100 ml of medium. However, the addition was made in the reaction vessel and only seconds before the tissue was introduced. This step prepared the tissue and the substrate for the enzymatic reaction with the peroxidase enzyme.

The enzymatic reaction was initiated by adding 2.0

ml of 0.3% H_2O_2 per 100 ml of medium for 20 minutes at room temperature. The sections were briefly removed from the medium for the addition of H_2O_2.

After the enzymatic reaction, sections were transferred, without washing, to a refrigerated stabilization solution for twenty minutes at 4^oC for twenty minutes. Each 100 ml of this solution contained 45 ml of distilled water, 50 ml absolute ethanol, nine grams of sodium nitroferricyanide and 5 ml of pH 3.3 buffer.

The sections were rinsed in distilled water, air dried and stained with hematoxylin and eosin. An effort was made to reduce the nonspecific activity with methanol and hydrogen peroxide (64, 65). This method was reported to reduce endogenous activity and allow only the specific activity of the added reagents to initiate and influence the reaction.

CHAPTER IV

RESULTS

Marked differences were seen in the clinical response and lung lesions and in the number, integrity and location of *Pasteurella* species in the lung lesions of calves that were challenged after vaccination with *P. multocida* or *P. haemolytica* and control calves challenged after sham vaccination with PBS. There were similar but less marked differences between calves vaccinated with *Pasteurella* species by aerosol versus the subcutaneous route. Four of the control calves challenged with *P. haemolytica* died as a result of challenge; none died as a result of the *P. multocida* challenge. Following intrapulmonic challenge with either *P. haemolytica* or *P. multocida*, calves exhibited clinical signs which were similar but the extent and duration were related to prechallenge treatment. Challenged calves were febrile, depressed and had a reduced appetite. A few animals had an excessive cloudy mucoid nasal discharge and a cough. Clinical signs persisted for 12-96 hours and in fatally affected animals persisted until death. The latter often became stiff, lame, developed one or more inflammed swollen joints,

and had painful grunting respiratory movements with severe respiratory distress. Lesions in the thorax of animals that survived four days after challenge included focal or diffuse serofibrinous pleuritis, marked regional lymphadenitis and, at the site of the challenge inoculation, a unifocal pneumona qualitatively typical of spontaneous acute pneumonic pasteurellosis. The severity of the clinical signs and thoracic lesions provided a basis for estimating resistance of each animal or treatment group of animals to challenge with the *Pasteurella* species.

Control animals sham vaccinated with aerosol or subcutaneously administered PBS and subsequently challenged with *P. haemolytica* (Table I, Appendix) exhibited clinical signs for 48-96 hours. Two of the animals were dead 20 hours post challenge and two others were dead 44 hours post challenge. These animals had diffusely edematous lungs with widespread petechia and ecchymoses in the visceral pleura and lung parechyma. Edema involved lung parenchyma, interlobular septa and pleura. Areas 15-20 cm diameter surrounding injection sites were more intensely edematous, more deeply congested and firm. The visceral and to a lesser extent the parietal pleura were often diffusely dull, granular and hemorrhagic. Extensive edema, congestion, hemorrhage and sometimes areas of necrosis were present in thoracic lymph nodes.

Control calves that survived the challenge had large focal pneumonic lesions, from 4x4x7 cm to 5x7x11 cm, which invaded surrounding lung parenchyma and interlobular connective tissue beyond the intensely pneumonic focus. The inflammatory process in the interlobular septa extended as much as 10 cm beyond the focus of parenchymal inflammation. Fibrinous pleuritis affecting the visceral and parietal pleura was diffuse or involved areas up to 16 cm in diameter surrounding the injection site. Calves in this group were judged not resistant on the basis of clinical response and gross pathologic features. Tissue sections from the control animals which died as a result of challenge contained large numbers of bacteria existing singly or in small groups in extracellular locations evenly distributed throughout (Figure 1). Microcolonies were observed in the tissue in some of the animals (Figure 2). In control calves killed four days after challenge there were large numbers of bacteria predominately in extracellular locations, but occasionally groups of bacteria existed in phagocytic cells. Nearly all bacteria observed had discrete bacillary form but occasionally phagocytic cells contained small amounts of brightly fluorescent, amorphous material without distinctly bacillary form; this material was interpreted to be bacterial antigen representing lysing bacilli.

Calves vaccinated with aerosol or subcutaneously

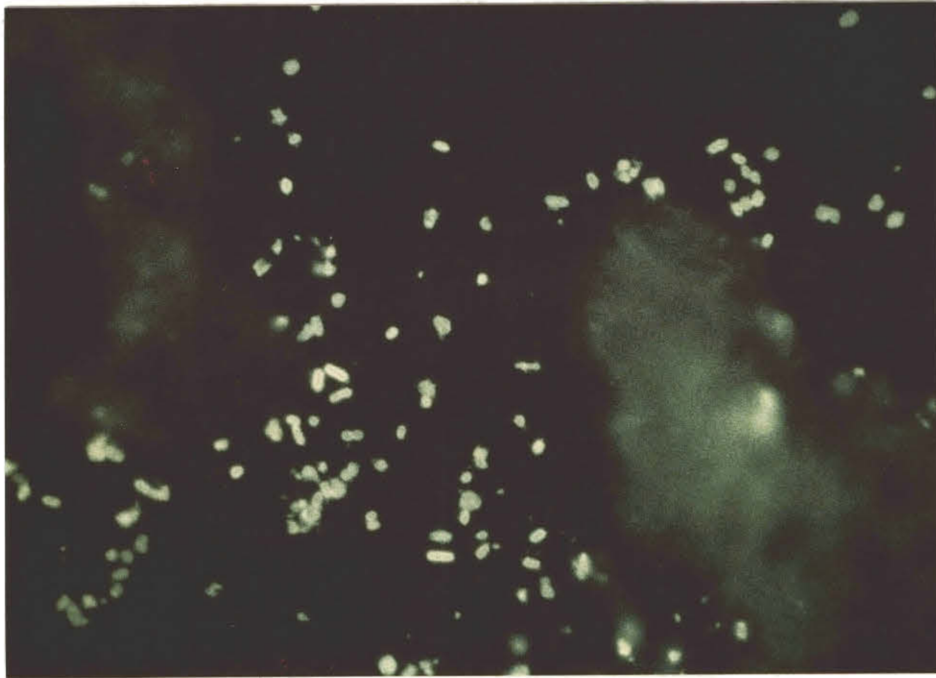

Figure 1. Extracellular Bacteria in the Lung Tissue of a Susceptible Calf. Mag. x 500

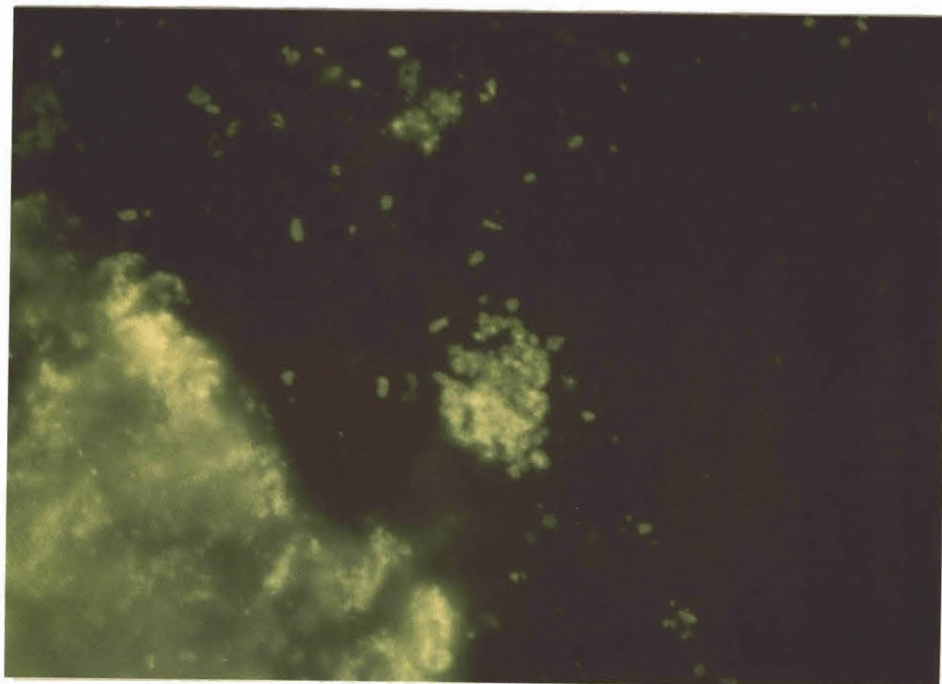

Figure 2. Microcolony of Bacteria in the Lung Tissue of a Susceptible Calf. Mag. x 500

administered P. haemolytica and subsequently challenged with P. haemolytica exhibited clinical signs which generally subsided within 24 hours. None of the animals died or showed evidence of systemic spread of infection. Vaccinated calves had focal lung lesions that were smaller than control calves ranging from 1.5x2x1.5 cm to 4x5x3 cm. The focus of pneumonia was surrounded by thick, edematous interlobular septa that essentially circumscribed the pneumonic focus. A zone of edema around the lesion was usually less than that present in the control calves extending 2-5 cm beyond the periphery of the pneumonic lesion. Some vaccinated calves had areas of fibrinous pleuritis limited to a 6-8 cm diameter area surrounding the injection site; others had no pleuritis. All animals vaccinated with P. haemolytica were judged resistant to challenge on the basis of clinical response and gross pathologic features. Lung lesions from calves vaccinated by subcutaneous injection of P. haemolytica contained fewer (less concentrated) organisms than the controls; a greater proportion of organisms was located intracellularly and there was considerably more fluorescing amorphous material present than in control calves. Some phagocytic cells contained many organisms but many contained only a few. A few scattered microcolonies were present in the lesions of two of the animals.

Lung lesions from calves vaccinated by aerosol of *P. haemolytica* contained sparsely distributed foci of fluorescence which were predominantly intracellular. Nearly all fluorescence was composed of amorphous, nonbacillary antigenic material. Stainable bacteria in these animals tended to be localized in small foci compared to diffusely distributed organisms present in control calves.

Results with *P. multocida* were similar to those obtained in the *P. haemolytica* experiments, however, there was more variation among animals of a group. Control calves sham vaccinated either by aerosol or subcutaneously with PBS and subsequently challenged with *P. multocida* (Table I, Appendix) showed clinical signs for 24-72 hours. All control calves survived the 96 hour post challenge interval. Lesions in the lungs of these calves were similar to the lesions in control calves challenged with *P. haemolytica*. Each lung of these calves had a single, large, intensely pneumonic focus ranging from 3x5x6 cm to 6x6x8 cm. The borders of the lesions blended into a surrounding zone of congested, edematous parenchyma. Markedly thickened, yellow, edematous interlobular septa often extended as much as 6-8 cm beyond the focus of parenchymal consolidation. Fibrinous pleuritis, chiefly visceral, existed in areas as great as 8-10 cm in diameter overlying the pneumonic focus. Pleuritis tended to be less extensive than in

calves challenged with *P. haemolytica*. Calves in this group were judged not resistant on the basis of clinical response and gross pathologic features. Tissue sections from 12 of the 13 control calves contained large numbers of bacteria in predominantly extracellular location. Occasional groups of bacteria were intracellular; most intracellular organisms were in bacillary form. One control calf had appreciable numbers of intact and degraded intracellular bacteria.

Animals vaccinated with *P. multocida* by subcutaneous injection exhibited mild clinical signs that subsided 24-48 hours after challenge. The lung lesions were smaller than lesions in control calves, from 2x2x1.5 cm to 3x4x5 cm, had well circumscribed borders and little surrounding parenchymal or interlobular edema. Pleuritis was either absent or limited to the site of needle puncture in the visceral pleura. Animals in this group were judged to be resistant on the basis of clinical signs and gross pathologic features.

Findings in tissue sections from these calves were somewhat variable. In three of the five calves there were masses (colonies) of extracellular fluorescing bacillary forms in small foci of apparently necrotic lung; the foci were surrounded by narrow zones of phagocytized bacillary forms and amorphous antigen and were separated by lung tissue that did not contain

fluorescent material. While the other two calves in this group had a similar distribution and arrangement of microorganisms, one had a considerably broader zone of phagocytized organisms surrounding necrotic foci and the other calf had more numerous extracellular organisms in the central area of the foci.

Calves challenged following vaccination by aerosol of *P. multocida* for 15 minutes showed mild clinical signs and were normal 24 hours after challenge. Four of five calves in this group had pneumonic foci which were small (1.5x1.5x2 cm to 3x3x6 cm) and well delineated by thick edematous interlobular septa at the periphery of the pneumonic focus. One calf had lung lesions which were larger and less well circumscribed. The lesions in each lung of the latter calf were judged not resistant; all other calves were judged resistant to challenge.

Lung sections contained only small numbers of bacteria and small quantities of amorphous antigen. Nearly all bacillary or amorphous fluorescent material was intracellular and was confined to small, irregular poorly demarcated foci containing occasional bacillary forms and considerable amorphous material. The surrounding tissue was free of fluorescence. One of the two sections from one calf contained an area in which there were large numbers of extracellular bacteria, singly and in groups, and no intracellular bacteria.

The surrounding tissue did not contain specific fluorescence. The one calf in the group judged not resistant on the basis of the gross appearance of the lung lesions was not distinguishable from other calves in the group when examined by the fluorescent antibody technique.

Two calves vaccinated for eight minutes with an aerosol of _P. multocida_ responded to challenge uniformly. The mild clinical signs resulting from challenge abated within 24 hours. While the clinical signs in these calves were mild, the gross lesions in the lungs resembled those of animals judged not resistant to challenge. Sections of lungs had discrete areas of irregular size (Figure 3) and shape that contained small numbers of extracellular bacillary forms and greater quantities of intracellular amorphous (degraded) antigen. The intervening tissue was free of intracellular antigen but contained a few extracellular bacteria, many fewer than control animals.

The two calves vaccinated for three minutes with an aerosol of _P. multocida_ and challenged showed clinical signs for approximately 48 hours post challenge. The clinical signs and lung lesions in these calves were judged to represent nonresistance. The distribution and location of bacteria in these two calves differed. One animal had discrete areas of extracellular bacteria bordered by phagocytic cells

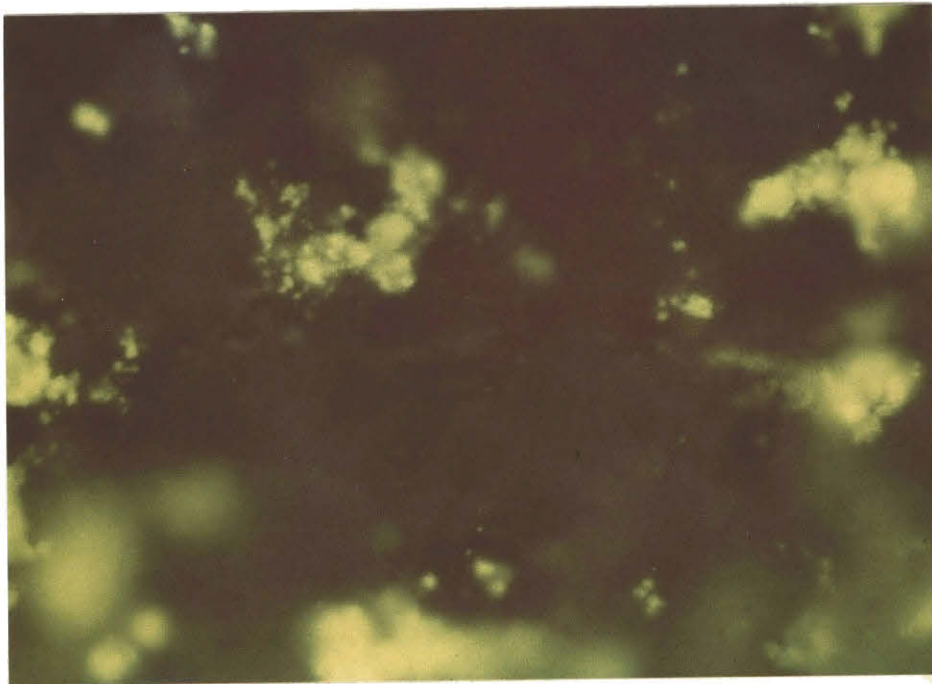

Figure 3. Extracellular Intact Bacteria and Intracellular Fluorescence in Calf Vaccinated with an Eight Minute Aerosol of *Pasteurella multocida*.
Mag. x 500

containing bacillary forms and amorphous antigen. These areas were separated by intervening tissue which did not contain specific fluorescence. The other calf had bacillary forms scattered diffusely throughout the tissue with many intact bacillary forms in both intracellular and extracellular locations. Tissues from this calf had greater numbers of intracellular bacteria than seen in control animals and many more extracellular bacteria than present in calves vaccinated by the subcutaneous route.

The calves in the IBR-*P. haemolytica* experiment received no vaccination prior to challenge. They had clinical signs which persisted for the four-five days until the calves were killed. Lesions in these calves included petechia of the visceral and parietal pleura, edematous, congested and hemorrhagic lungs. The pneumonic lesions, centered at the injection site, extended to involve nearly the entire diaphragmatic lobe and had irregular, poorly defined borders. These calves had more diffuse lung lesions and more extensive pleuritis than calves not concurrently infected with IBR virus. The distribution and number of bacteria in these calves differed from calves not infected with IBR virus. Tissue sections contained extracellular bacteria in large numbers (Figure 4) distributed diffusely throughout the lesions; intracellular bacteria were only rarely observed. The extracellular bacteria were seen

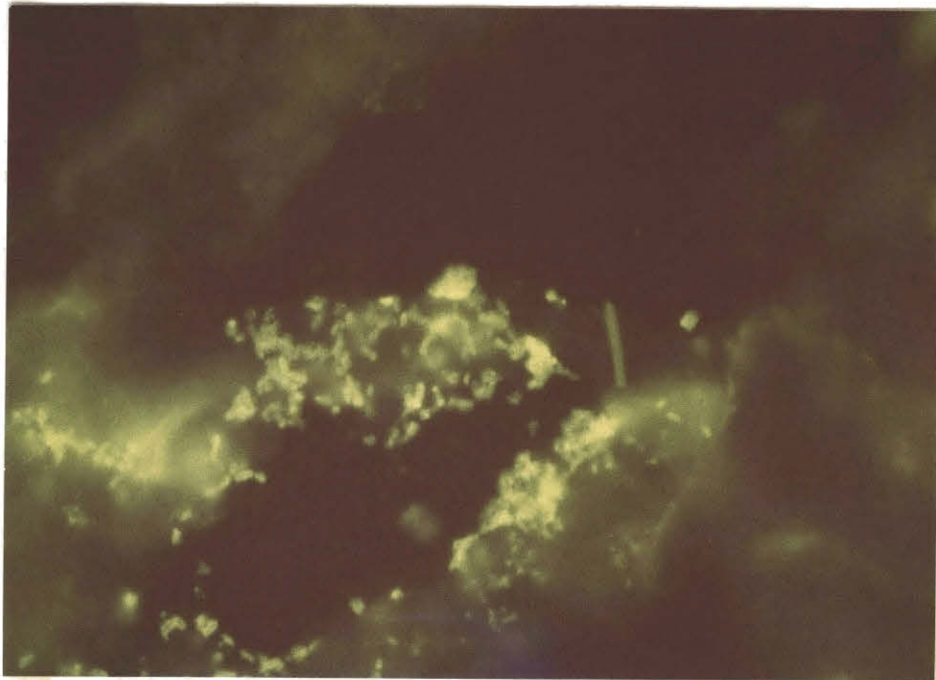

Figure 4. Numerous Extracellular Bacteria in the Lung Tissue of a Calf Inoculated with IBR Virus Prior to *Pasteurella haemolytica* Intrapulmonic Challenge. Mag. x 500

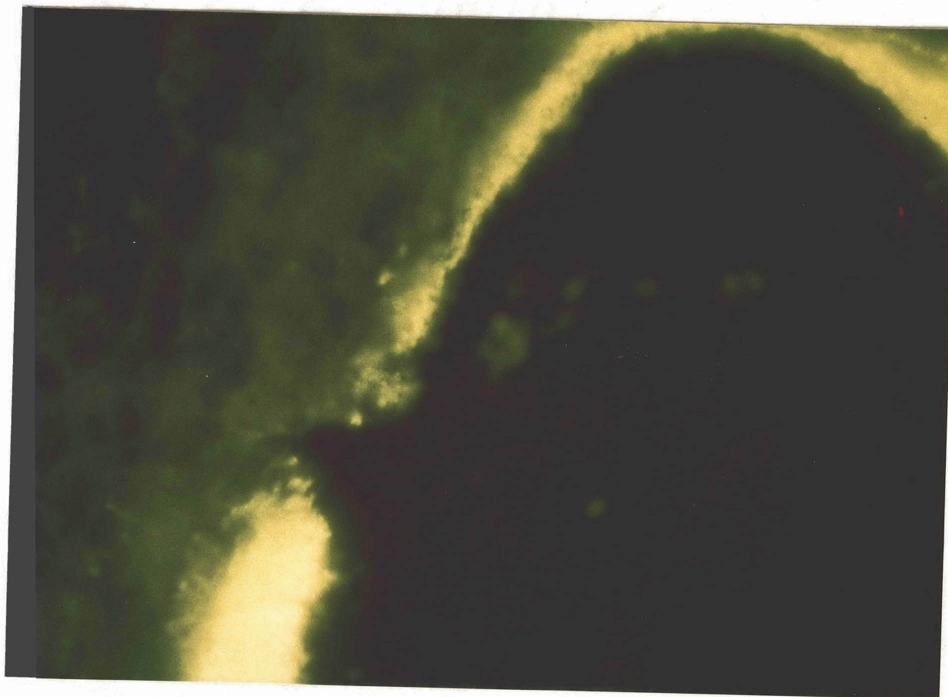

Figure 5. Extracellular Bacteria Lining the Alveolar Walls in a Calf Inoculated with IBR Virus Prior to *Pasteurella haemolytica* Intrapulmonic Challenge. Mag. x 500

as concentrations lining the alveolar walls in two of the three calves (Figure 5).

The electron microscopic study was done to more precisely determine the location of the bacteria in the lung tissue. The intracellular or extracellular location of the bacteria would be easily determined due to the thin sectioning of the tissue samples. The irregular location of the bacteria observed with the fluorescent antibody technique was seen in the samples prepared for the electron microscope in that bacteria were not seen in most of the sections. Bacteria were seen in only two of the samples, one from a resistant calf and one from a susceptible calf. The lung tissue from the lesion sites did undergo degenerative changes. The most evident changes were hemorrhage and the presence of very large amounts of fibrin in the intercellular spaces. Destruction of the alveolar wall was also seen in the loss of cytoplasm from type one alveolar cells normally seen lining the surface of the alveolar septum. The remaining cytoplasm of type one and type two cells contained swollen mitochondria with ruptured christae and expanded endoplasmic reticulum. Pyknosis and karyorrhexis were evident in the nuclei in most cells of the tissue. The basal lamina appeared to be intact in the micrograph but the hemorrhage seen indicates the basement membrane was not intact in all places (Figure 6).

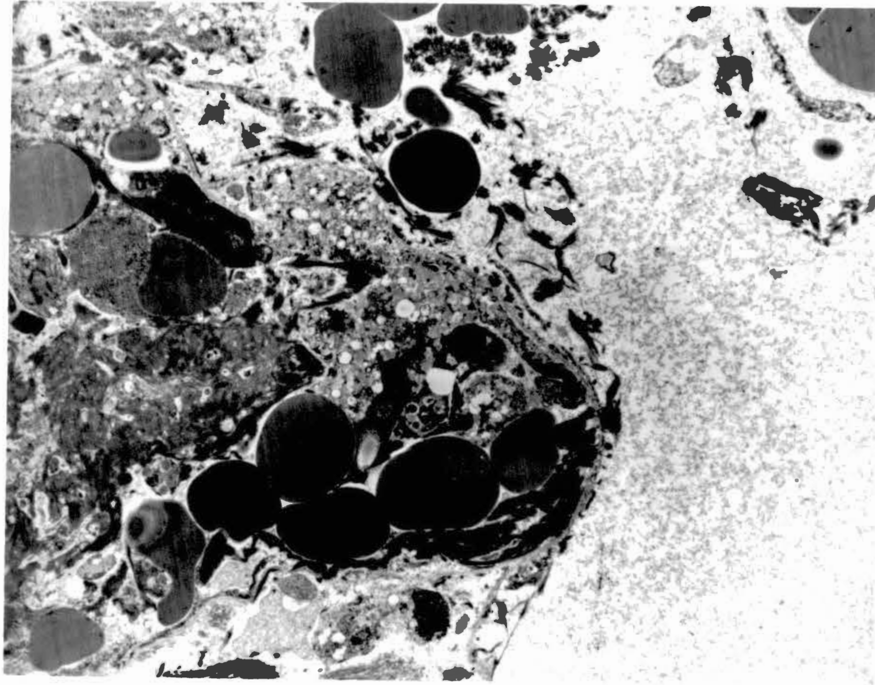

Figure 6. Electron Micrograph Showing the Loss of Type I Alveolar Cell Cytoplasm, the Expanded Endoplasmic Reticulum, Fibrin Deposition and Hemorrhage in a Susceptible Calf.
Mag. x 4700

Bacteria were observed in extracellular locations in the sample from the susceptible calf (Figure 7). The bacteria were grouped in clumps or microcolonies in areas of the lung in which the cells appeared to be dead. There were several of groups of bacteria seen in this calf from the center of the lesion. Bacteria were not seen in other samples from this calf or in samples from other susceptible calves.

Bacteria were seen in one sample from a resistant calf surrounded by a cellular membrane (Figure 8). The cell type or the integrity of the cell can not be determined due to the lack of sufficient cellular detail and stuctures. The membrane is probably not the plasma membrane since there are no recognizable cytoplasmic organelles enclosed by the membrane, the membrane may be that of a phagocytic vacuole. Other structures near the membrane enclosed cluster of bacteria, such as mitochondria, suggest that it is intracellular. Alternatively, lysis of the cell containing the phagocytic vacuole may have occured with release of the vacuole and other cytoplasmic organelles into the extracellular environment. The micrograph also contains a few single bacteria which may be extracellular. The disruption of the membranes in the area hinders determination of the precise relationship of the bacteria to the pulmonary cells. Bacteria were also seen in a group in the middle of an alveolus in this calf not surrounded by any mem-

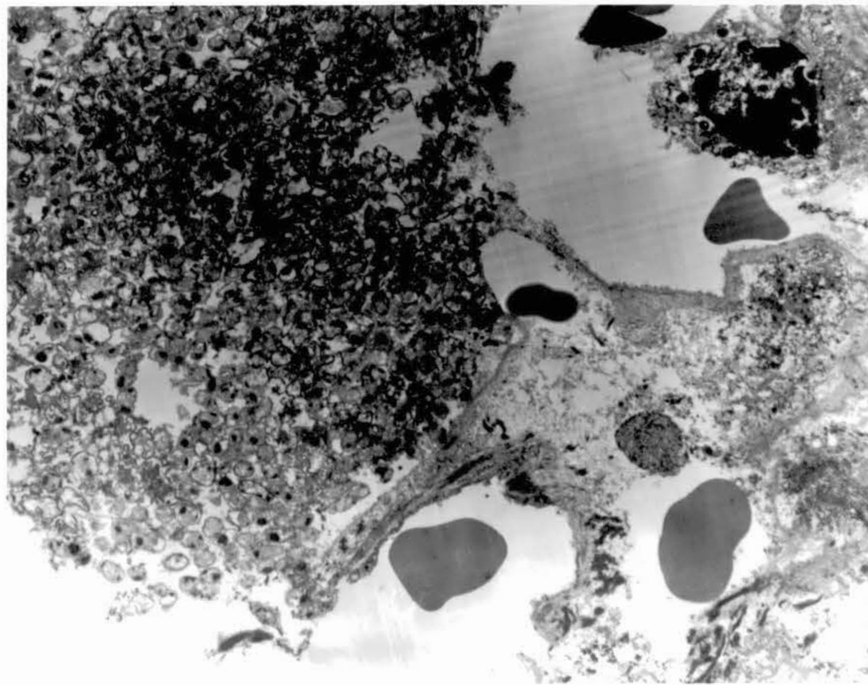

Figure 7. Electron Micrograph of Lesion Site with Extracellular Bacteria in an Area of Hemorrhage and Lack of Tissue Structure Indicating Tissue Necrosis.
Mag. x 4700

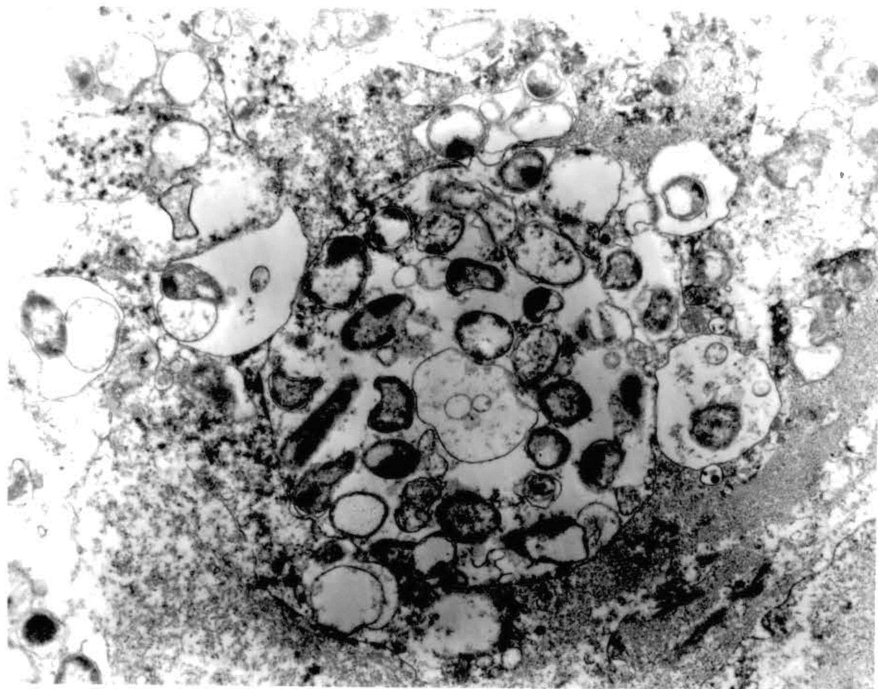

Figure 8. Bacteria Surrounded by Membrane in the Lung of a Resistant Calf. The Membrane Appears to be of a Phagocytic Vacuole and not the Plasma Membrane.
Mag. x 7500

brane (Figure 9). There were what appeared to be degenerating mitochondria mixed with the bacteria. These mitochondria may have come from cell debri from parenchymal cells lysed as a result of the infectious process or from a phagocytic cell which had engulfed the bacteria and then lysed, releasing the bacteria in a group. Plasma cells were frequently seen in the tissue of resistant calves in the alveolar septa with granular endoplasmic reticulum which was expanded to a larger than usual diameter indicating the production of large amounts of protein, presumably antibody.

The electron microscopic study did not fully substantiate the results from the fluorescent antibody technique study due to the small numbers of bacteria seen in the samples. Evidence of intracellular degraded bacteria in the phagocytic cells, such as portions of bacterial cell walls, was not seen in any of the tissue samples examined. Bacteria were not seen in any pulmonary cell which was detailed enough to determine the cell type or integrity and the fate of any bacteria which appeared to be membrane bound could not be determined.

The mechanisms of antiserum dilutions, substrate compounds and specific activity in the immunoperoxidase procedure for proper use with this system were not established. The dilutions of antiserum and conjugate, the substrate, and the method of blocking endogenous activity were not sufficient to demonstrate bacteria in

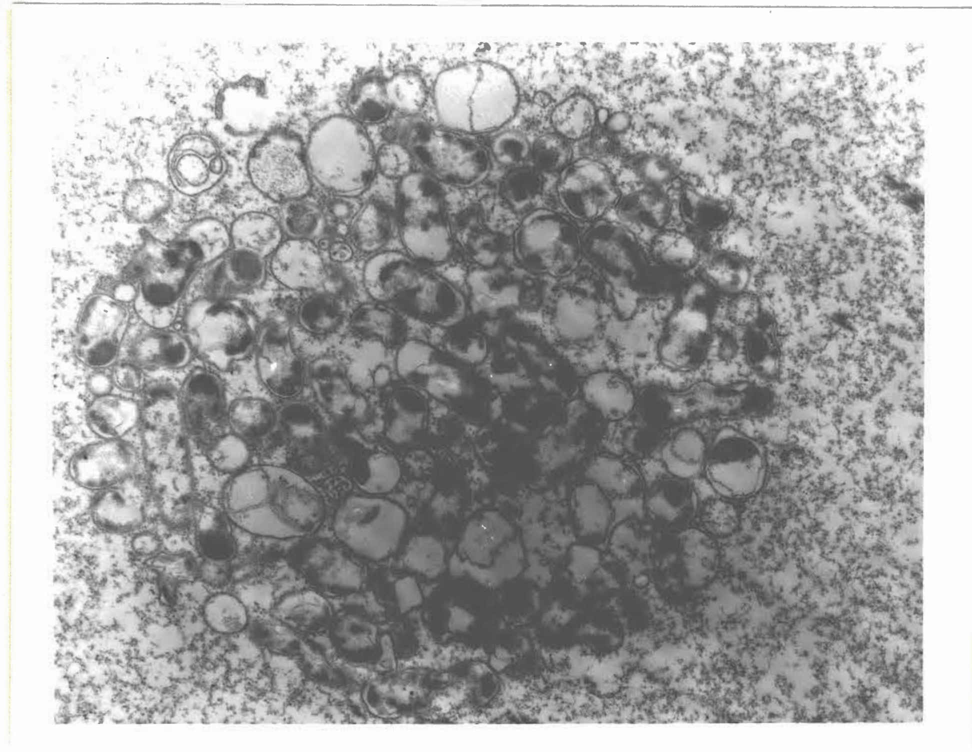

Figure 9. Bacteria in Clump with Cellular Debri in Lung of a Resistant Calf. The Bacteria Could Have Been Released from a Ruptured Phagocytic Cell. Mag. x 7500

the lungs of calves. Both the tissue samples and smears of the bacteria contained high levels of endogenous activity. This activity resulted in the peroxidase reaction proceeding at an uncontrolled rate in the procedure. The substrate was converted to product which was deposited over the entire tissue sample or bacterial smear. This endogenous activity was much greater than the specific activity produced by the horseradish peroxidase labeling the antigen in this procedure. The endogenous activity was not controlled with methanol and hydrogen peroxide treatment of the samples prior to labeling with the specific antibody-antibody enzyme label. Changing the substrate to 3,3'-diamino benzidine did not result in any improvement as the reaction still proceeded in an uncontrolled manner. The end product of the reaction was deposited over the entire surface of the tissue without any selective sites determined as foci of bacterial antigen.

CHAPTER V

DISCUSSION

The distribution, relative numbers, and integrity of transthoracically administered bacteria as demonstrated by the fluorescent antibody technique varied according to pre-challenge treatment of the calves. The generally consistent results among animals within treatment groups and consistent differences between treatment groups indicate pre-challenge treatment to be the major factor responsible for the observed results.

Animals vaccinated by 15 minute exposure to an aerosol of either *P. haemolytica* or *P. multocida* were the most resistant to challenge. Post-challenge clinical response and gross lesions within the lungs were notably less severe than those of other treatment groups. Findings in sections of lung subjected to the fluorescent antibody technique also indicated increased resistance: they contained a low concentration of organisms, most organisms were phagocytized and most organisms were being degraded. The lungs of animals vaccinated subcutaneously generally had greater numbers of organisms than did the group vaccinated with the

aerosol method and more organisms retained bacillary form, and while the majority were intracellular, fewer were degraded.

Observations in animals vaccinated with an aerosol of P. multocida for three or eight minutes indicated moderate and inconsistent degrees of resistance. In neither instance did the resistance approach that in calves vaccinated by aerosol for 15 minutes, however they did approach resistance manifested in animals vaccinated subcutaneously.

Animals sham vaccinated by either aerosol or subcutaneous administration of phosphate buffered saline were least resistant to challenge. They manifested more severe clinical disease; four succumbed to challenge due to acute, diffuse pulmonary inflammation and edema and septicemia; post-challenge lung lesions were more severe than in vaccinates. Microscopically, observations in the lungs of these animals indicated considerably lower efficiency in pulmonary defense mechanisms as evidenced by the high concentration of bacteria present, the relative sparsity of phagocytized organisms and the sparsity of degraded bacilli.

Wilkie (66) reported more severe disease in animals challenged following subcutaneous vaccination with formalin killed P. haemolytica in Freund's complete adjuvant than in nonvaccinated animals. Those results should not discourage attempts to perfect effective

vaccines to *P. haemolytica* or *P. multocida*. Methods used in the current study indicate resistance of the lung to the challenge inoculum is remarkably increased by both the aerosol and the subcutaneous vaccination procedures applied.

On the basis of the profusion of bacteria and the rare occurence of phagocytized organisms in calves concurrently infected with infectious bovine rhinotracheitis virus and *P. haemolytica* defense mechanisms appeared less effective even than those in control calves sham vaccinated with phosphate buffered saline. Impaired defense after viral infection might be caused by viral effects on phagocytic efficiency of pulmonary alveolar macrophages, hence, diminished bacterial clearance (67, 68), or induction of pulmonary edema or other factors that provide a pulmonary environment more favorable for the growth of organisms.

The net effects of vaccination were a reduction in the numbers of demonstrable organisms present in the challenge lesions and greater efficiency in the phagocytosis and degradation of bacilli in the challenge lesions. These events, discernible at the microscopic level, were reflected grossly by limitation of the invasiveness and extension of the inflammatory process resulting from the deposition of challenge culture into the lung parenchyma; hence there was a smaller, more well circumscribed focus of pneumonia. Clinically,

there was less severe, less persistent, post-challenge illness and inhibition of systemic spread of the pulmonary infection. The results infer that vaccination by delivery of the live antigen directly to the respiratory tract, including the lung, enhances the ability of the lung of vaccinated animals to inhibit growth of the organisms and spread of the infection within the lung and that it inhibits systemic spead of the infection. The mechanisms responsible for enhanced control of the infection in the lung were not determined.

Although P. haemolytica has ben reported to be highly toxic to pulmonary alveolar macrophages in vitro (69, 70, 71) this study indicates that macrophages of vaccinated animals survive in vivo and that their ability to engulf and destroy or degrade the living organisms was greatly enhanced. Whether vaccination caused macrophage activation, opsonization, or was due to combinations thereof, or to other factors, remains to be determined. Lysis of bacteria was recognized only in organisms that had been engulfed by phagocytic cells. The evidence then suggests that a major factor in control of infection was enhancement of phagocytosis and intraphagocytic killing of the organisms.

The electron microscopic study was hindered by the very small sample size required for proper fixation and sectioning. The irregular pattern of distribution of bacteria seen with the fluorescent antibody technique

indicates that taking a very small sample would result in a chance selection of tissue with bacteria that could be studied. This problem could be circumvented by taking and examining a larger number of samples than was done in this study. The frozen samples taken for the fluorescent antibody technique were many times larger in width and depth than samples prepared for the electron microscope. This larger volume of tissue made finding bacteria or bacterial antigen much more likely.

The immunoperoxidase procedure has usually been applied to work looking for single chemical compounds or antigenic components. The system with *P. haemolytica* was a much more complex system with probably many more antigens involved and therefore more antibody-antibody enzyme labels attached to the tissue. This itself would cause an increase in the activity of the system, but added onto that were enzymes from the bacteria, the normal pulmonary enzymes, extracellular enzymes released from the tissue damage to the lesion site and the enzymes of the inflammatory cells responding to the lesion, all of which may have peroxidase activity. Such a complex of enzymes from many sources, presumably resulted in an uncontrolled reaction which overwhelmed the specific reaction and resulted in greatly increased substrate deposition which did not coincide with the presence of bacteria or bacterial antigen. The usual methods for inhibiting the endogenous activity of a

system were not sufficient to control this reaction in this case. Perhaps the use of different dilutions of serum or peroxidase conjugated antiglobulin would result in a system in which the endogenous activity of the tissue could be controlled with the use of inhibitors.

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APPENDIX

TABLE I

EXPERIMENTAL PROCEDURE; RESULTS OF EXAMINATION BY FAB

Vaccination or treatment methods	Number of calves	Bacterial reponse in the lungs of calves as determined by FAB
P. haemolytica subcutaneous injection	4	Numerous bacteria; intracellular both intact and degraded, moderate numbers extracellular.
P. haemolytica 15 minute aerosol	4	Few bacteria; predominantly intracellular and degraded, few extracellular.
PBS subcutaneous injection	4	Very numerous bacteria; almost entirely extracellular and intact.
PBS 15 minute aerosol	4	Very numerous bacteria; almost entirely extracellular and intact.
P. multocida subcutaneous injection	5	Moderately numerous bacteria; distributed extracellularly and intracellularly, both intact and degraded.
P. multocida 15 minute aerosol	6	Few bacteria; predominantly intracellular and degraded. Extracellular bacteria numerous in one calf.
P. multocida 8 minute aerosol	2	Moderate numbers of bacteria; predominantly extracellular and intact, rare intracellular intact.

TABLE I (Continued)

P. multocida 3 minute aerosol	2	Numerous bacteria; predominantly extracellular and intact, few intracellular intact bacteria.
PBS subcutaneous injection	5	Very numerous bacteria; predominantly extracellular and intact, few intracellular intact bacteria.
PBS 15 minute aerosol	6	Very numerous bacteria; predominantly extracellular and intact, very few intracellular intact bacteria.
PBS 8 minute aerosol	1	Very numerous bacteria; predominantly extracellular and intact, rare intracellular intact bacteria.
PBS 3 minute aerosol	1	Very numerous bacteria; predominantly extracellular and intact, rare intracellular intact bacteria.
IBR virus *P. haemolytica* challenge	3	Extremely numerous bacteria; extracellular throughout the tissue and lining the alveolar walls.

VITA

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