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Cell-mediated immune responses of cattle to _Pasteurella haemolytica_ and responses of goats to alternative routes of exposure to _Pasteurella haemolytica_

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Iowa State University, 1993

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Cell-mediated immune responses of cattle to

Pasteurella haemolytica and responses of

goats to alternative routes of exposure to

Pasteurella haemolytica

by

Brad M. DeBey

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GENERAL INTRODUCTION

Acute bovine pneumonic pasteurellosis is primarily caused by <u>Pasteurella haemolytica</u>. Currently, 16 serotypes (1-16) of <u>P</u>. <u>haemolytica</u> have been identified within two biotypes (A and T). <u>Pasteurella haemolytica</u> serotype 1, which is of the A biotype, is the type most frequently isolated from pneumonic bovine lungs (Frank, 1986). This bacterium is one of several etiologic agents associated with the bovine respiratory disease complex, and is considered to be the agent associated with severe morbidity and mortality of cattle affected with the bovine respiratory disease complex (Yates, 1982). Pneumonic pasteurellosis occurs primarily in calves, associated with stressful conditions such as weaning, transportation, and commingling of cattle from different sources.

Reproduction of pneumonic pasteurellosis by introduction of <u>P</u>. <u>haemolytica</u> into the respiratory tract is sometimes met with failure, although it has been stated that <u>P</u>. <u>haemolytica</u> can and does cause pneumonia by itself, without the assistance of other pathogens (Thomson, 1980). A blood-borne route of arrival of <u>P</u>. <u>haemolytica</u> to the bovine lung has been proposed (Thomas et al., 1989), although there has generally been scanty support of this hypothesis (Carter, 1954).

Humoral immune responses of cattle to <u>P</u>. <u>haemolytica</u>

have been extensively studied, and antibody responses to certain components of P. haemolytica have been proposed to be associated with protective immunity. The cell-mediated branch of the bovine immune response, however, has not been evaluated as a factor in protective immunity against P. haemolytica. The objectives of this study were i) to establish a caprine model of penumonic pasteurellosis by comparing the pulmonary responses to intratracheal, intravenous, and intratonsillar routes of inoculation with P. haemolytica, ii) to develop techniques and conditions necessary to evaluate cell-mediated immune responses to P. haemolytica, and iii) to assess the cell-mediated immune responses of cattle after vaccination or infection with P. haemolytica.

Dissertation format

This dissertation includes two manuscripts, the first of which has been published in Veterinary Research Communications. The second manuscript has been prepared for submission to Infection and Immunity. Review of the literature precedes the first manuscript, and a general summary and discussion follows the second manuscript. References cited in the manuscripts are listed at the end of each manuscript. Literature cited in the general introduction, literature review, and general summary and discussion appears in the same format as the first

manuscript, and is located after the general summary and discussion. Three appendices conclude the dissertation, with the first including additional methods that were used in Paper 1. The second appendix contains methods and results of antileukotoxin antibody titers from Paper 2. The third appendix includes data from preliminary studies of nasal colonization of calves by P. haemolytica. The nasal colonization studies were discontinued due to technical dificulties.

The Ph.D. candidate, Brad M. DeBey, was the principal investigator for each study.

LITERATURE REVIEW

Historical background

The naming of bovine pasteurellosis (Shirlaw, 1938) resulted from observations of two separate syndromes in cattle that were associated with Pasteurella species. It was known that Pasteurella species were associated with hemorrhagic septicemia and pneumonia in cattle (Jones, 1921). The two syndromes were classified separately, with bovine pasteurellosis characterized as the pneumonic form, and usually associated with P. haemolytica (Newsom and Cross, 1932).

Pneumonic pasteurellosis of cattle was often associated with transportation of cattle, hence, the names 'shipping fever', or 'transit fever' (Kinsley, 1915, Marshall, 1922, Hepburn 1925, Anderson, 1939). Numerous etiologic agents have been determined to be associated with bovine respiratory tract disease (Yates, 1982), however, P. haemolytica or P. multocida were regarded as the cause of clinical signs and death loss (Collier, 1968). Thomson (1980) stated that P. haemolytica can and does cause shipping fever by itself, although parainfluenza-3 virus (PI-3 virus), infectious bovine rhinotracheitis virus (IBR virus), bovine respiratory syncytial virus, or mycoplasmas may facilitate the process.

Virulence factors of Pasteurella haemolytica

Fimbriae   Fimbriae have been demonstrated on P. haemolytica grown in vitro (Morck et al., 1987) and on bacterial cells recovered in pulmonary lavage fluid from an experimentally infected calf (Morck et al., 1988). Fimbriae were proposed to promote adhesion of P. haemolytica to upper respiratory tract mucosal surfaces, and structures resembling fimbriae were demonstrated on P. haemolytica that were adherent to tracheal epithelium in a naturally infected calf (Morck et al., 1989).

Capsular polysaccharide   Electron microscopic studies (Gilmour et al., 1985, Morck et al., 1988) have demonstrated that Pasteurella haemolytica produces a glycocalyx (capsule) composed in part of serotype-specific polysaccharides (Adlam et al., 1986). Morck et al. (1988) demonstrated intimate association of encapsulated P. haemolytica to alveolar epithelium, proposing that capsular material may aid in adherence of the organism to alveolar epithelium.

Capsular polysaccharide of P. haemolytica released into lung alveoli in the early stages of infection may alter phagocytic cell function. After incubation of capsular polysaccharide of P. haemolytica with bovine neutrophils (Czuprynski et al., 1989) or alveolar macrophages (Czuprynski et al., 1991), a marked decrease in phagocytosis and killing of the organism was demonstrated.

Leukotoxin   Benson et al. (1978) first described in vitro cytotoxicity caused by P. haemolytica. The cytotoxic factor was later identified as leukotoxin, which is present in culture supernatants as a protein polymer with a molecular weight greater than 400 kilodaltons (kDa) (Chang et al., 1987). The protein that confers leukotoxic activity to the complex has a molecular weight of 102 kDa (Lo, 1990). Leukotoxin of P. haemolytica is a member of a family of toxins termed Repeats in Toxin (RTX) that are genetically related (Welch et al.,1992). The Escherichia coli hemolysin (HlyA) and the Bordetella pertussis adenylate cyclase/hemolysin (CyaA) are also RTX toxins.

Leukotoxin elaborated from P. haemolytica is a pore-forming cytolysin (Clinkenbeard et al., 1989) that causes lysis of bovine neutrophils (Baluyut et al., 1981), peripheral blood monocytes, lymphocytes and alveolar macrophages (Shewen and Wilkie, 1982), and platelets (Clinkenbeard and Upton, 1991). In lower, nonlethal concentrations, leukotoxin reduces functional capabilities of bovine neutrophils (Czuprynski and Noel, 1990), alveolar macrophages (Richards and Renshaw, 1986) and lymphocytes (Czuprynski and Ortiz-Carranza 1992).

Shewen and Wilkie (1983a) demonstrated leukotoxin production by all of the 12 recognized serotypes of P. haemolytica. The authors concluded that leukotoxin

production solely by serotype 1 was not the factor related to the nearly unique involvement of P. haemolytica serotype 1 in bovine pneumonic pasteurellosis.

Lipopolysaccharide   Pasteurella haemolytica lipopolysaccharide (LPS) is similar to LPS produced by other gram-negative bacteria, and is composed of lipid A, core oligosaccharide, and an antigenic polysaccharide chain (O antigen) (Confer et al., 1990). Biological properties of P. haemolytica LPS are similar to LPS of Escherichia coli, with intravascular injections of P. haemolytica LPS causing increased pulmonary arterial pressure and decreased cardiac output (Keiss et al., 1964).

The LPS of P. haemolytica probably contributes a major component to the inflammatory response that occurs in pneumonic pasteurellosis. Experimentally, administration of P. haemolytica LPS to calves induces release of prostaglandins, serotonin and histamine (Emau et al., 1984), and intrabronchial instillation of P. haemolytica LPS in sheep causes acute pneumonia (Brogden et al., 1984). However, Confer et al. (1986a) demonstrated that serum antibody response to P. haemolytica LPS in calves vaccinated for P. haemolytica is not important for resistance to experimental challenge with the organism.

Experimental production of pneumonic pasteurellosis

Controversy exists as to the methods of choice to

experimentally produce pneumonic pasteurellosis. Many techniques have been attempted, some successfully and others unsuccesfully, to reproduce the disease.

Intranasal inoculation of P. haemolytica was unsuccessful in producing disease in several studies. Horlein and Marsh (1957) exposed calves to nasal exudates from calves with natural cases of shipping fever, with no disease resulting from the inoculations. Carter (1956) inoculated 21 calves intranasally with P. haemolytica; although not all calves were necropsied after inoculation, pneumonic lesions from which P. haemolytica was isolated were present only in two calves.

Attempts of experimental infections using intratracheal or intrabronchial injection of P. haemolytica have been reported to have variable results of producing pneumonia. Early studies often indicated that intratracheal injection of P. haemolytica did not consistently produce pneumonic lesions (Carter, 1954) (Gale and Smith, 1958) (Heddleston et al., 1962). In the more important of these studies (Gale and Smith, 1958), 16 calves were intratracheally inoculated with up to 1.8×10^{11} CFU of P. haemolytica, with no signs of disease occurring, and pneumonic lesions were not found in any of the 16 calves at necropsy.

More recent studies, however, indicate more consistent production of pneumonia with intratracheal inoculation of P.

haemolytica. Gibbs et al., (1984) reproduced pneumonic pasteurellosis by intratracheal inoculation of conventional calves with 2.0 X 10^{10} to 8 X 10^{12} CFU of P. haemolytica given five inoculations during three consecutive days. At necropsy, approximately 70% of the anterior lung lobes were pneumonic in four of four calves inoculated. Ames, et al. (1985) inoculated calves intratracheally in groups of three with varying doses of logarithmic growth phase P. haemolytica cultures. At necropsy, pneumonia was present at up to 38.3% of the total lung volume in calves inoculated with 1 X 10^9 CFU of P. haemolytica. Wilkie et al. (1980) produced pneumonic lesions in 57.1% of calves by intrabronchial inoculation of 2 X 10^6 or 2 X 10^7 CFU of P. haemolytica.

Authors have cautioned readers about intratracheal inoculation techniques often used to produce pneumonic pasteurellosis. Gilmour et al. (1975) stated that studies in which large doses of bacteria were given directly into the lung should be treated with reserve, since very large numbers of organisms might be expected to produce a pathological change. Jericho (1987) stated that inoculation of animals by methods that are unnatural (i.e. intratracheal injection) may produce tissue responses with visual appearances similar to disease occurring in natural conditions, but that studies in which emulation of natural disease is needed should use methods as natural as possible, such as aerosol exposure with

P. haemolytica.

The unnatural production of disease by intratracheal inoculation of large numbers of organisms led scientists to attempt to produce pneumonic pasteurellosis by aerosol exposure of the respiratory tract with P. haemolytica. Saunders and Berman (1964) aerosol-exposed 6 calves to P. haemolytica after various stressors, with no significant responses occurring. Gilmour et al. (1975) inoculated colostrum-deprived lambs with approximately 1 X 10^5 CFU of P. haemolytica by aerosolization, with pneumonic lesions developing in only 4 out of 9 lambs. Frank (1979) stated that aerosol exposure of calves to P. haemolytica either resulted in no reaction, or only a one-day febrile response.

Panciera and Corstvet (1984) described a method of production of pneumonic pasteurellosis in which 2-5 ml of a P. haemolytica inoculum was placed into the caudal lung lobes of cattle by transthoracic injection. This technique caused focal expanding pneumonia in 20 of 23 calves, with lesions up to 15 cm in diameter. The three remaining calves died of septicemia. This technique was subsequently used as a model of induction of pneumonic pasteurellosis in several studies evaluating protection by various immunization techniques (Confer et al.,1984, Confer et al., 1986b, Confer et al., 1987).

Pneumonic pasteurellosis has been produced by

inoculation of calves with PI$_3$ virus (Baldwin et al. 1967) or bovine herpesvirus 1 (BHV-1) (Jericho et al., 1976), followed by inoculation with P. haemolytica. However, Frank (1979) summarized several studies reporting a range of clinical responses, from none to severe respiratory distress resulting from combined exposure of cattle to PI$_3$ virus or BHV-1 and P. haemolytica. Studies in which viral agents are used in conjunction with P. haemolytica are likely to have clinical, and possibly lung responses resulting from P. haemolytica confounded with responses resulting from viral disease.

Intravenous inoculation of calves with P. haemolytica did not result in pneumonic lesions in two studies (Carter, 1956, Horlein and Marsh, 1957). In a later study, however, Thomas et al. (1989) produced pneumonia in 5 of 5 calves that were inoculated intravenously with approximately 1 X 10^8 CFU of P. haemolytica.

Alternative pathogenesis of initiation of pneumonic pasteurellosis

Because of the difficulty often encountered in reproducing pneumonic pasteurellosis by methods that mimic natural conditions, the study by Thomas et al. (1989) provided evidence that events involved in initiation of the disease may be misunderstood. It is generally accepted that initiation of pneumonic pasteurellosis begins with aerosolization of P. haemolytica into the lung, a concept

that has been summarized by Frank (1979). Grey and Thomson (1971) proved that P. haemolytica is present in the tracheal air of calves that have nasal passages colonized with the organism, and Jubb and Kennedy (1970) state that the pneumonic lesions start at the level of the respiratory bronchiole, suggesting that aerosolization of the organism is involved in initiation of pneumonia.

Evidence to contradict the airway-route of initiation of pneumonic pasteurellosis was introduced when Thomas et al. (1989) described the production of pneumonic lesions in calves challenged intravenously with P. haemolytica. In this study, calves inoculated intravenously with P. haemolytica A1 developed pneumonic lesions with distribution patterns and gross and microscopic lesions very similar to those occurring in natural cases of pneumonic pasteurellosis. Furthermore, Anderson et al. (1991) isolated P. haemolytica from livers, spleens and mesenteric lymph nodes of cattle that were inoculated intrabronchially with P. haemolytica, providing evidence of bacteremia.

The ruminant tonsil is a possible site of invasion of P. haemolytica. The sinus of the palatine tonsil of ruminants opens into the pharynx, where colonization of P. haemolytica is likely to occur under certain conditions (Frank, 1992). During conditions of colonization of the nares and nasopharynx, Pasteurella hamolytica might gain access into

the tonsil. Smith et al. (1983) demonstrated that cattle can
generate carrier states where P. haemolytica is present in
the upper respiratory tract, yet elicit no humoral antibody
response, which might allow long-term colonization of the
tonsil.

Pasteurella haemolytica biotype A has been isolated from
the tonsils of sheep under natural conditions (Al-Sultan and
Aitken, 1985). After experimental infection of the palatine
tonsils of calves with P. haemolytica A1, the organism could
be isolated from the tonsils for a duration of at least three
weeks (Frank and Briggs, 1992). The authors of this study
suggested that the bovine tonsil is a likely location where
P. haemolytica resides in healthy cattle.

Once residing in the tonsil, P. haemolytica might invade
the lymphoid tissue and gain access to the lung via the
vascular system. Mycobacterium paratuberculosis (Momotani et
al., 1988) and Brucella abortus (Ackermann et al., 1988) are
endocytosed by the specialized follicle-associated epithelium
of lymphoid tissue and transported to underlying phagocytic
cells, allowing a probable portal of entry. Such a portal of
entry has not been demonstrated for P. haemolytica, however.

Evidence of invasion of P. haemolytica was demonstrated
in a study where two of 12 calves that had P. haemolytica
inoculated into their tonsillar sinuses developed pneumonia
(Frank et al., 1991). Calves also had swollen joints and

necrosis of retropharyngeal lymph nodes which was
interpreted as evidence of invasion by P. haemolytica.
Although the above theory indicates that P. haemolytica might
invade the tonsil and gain access to the lung via the
vascular system, it has not been proven.

Protective immunity to Pasteurella haemolytica

Bacterins   Vaccination studies of calves with P.
haemolytica bacterins were often reported as clinical studies
(Amstutz et al.,1981, Handy and Trapp, 1964, Hamdy et al.,
1965), in which reduction of clinical signs of disease was
not demonstrated. Palotay et al. (1963) vaccinated calves
prior to weaning with several different P. haemolytica
bacterins, demonstrating significant reduction in morbidity
in vaccinated calves after weaning, although the incidence of
disease was almost 10% in the vaccinated calves. There was
no mention of adjuvants used in any of the bacterins.
Beneficial effects of vaccines demonstrated in field studies
may be influenced by concurrent diseases other than
pasteurellosis, and Panciera and Corstvet (1984) emphasized
the value of models of disease to interpretable studies.

Jericho et al. (1990) vaccinated calves twice
intramuscularly with a formalin-killed P. haemolytica
bacterin with no adjuvant. Calves were later aerosol-
challenged with BHV-1 and P. haemolytica, resulting in a mean
percentage of pneumonic lung of 1.2% in vaccinated calves,

versus 25.2% in control calves. The authors of this study stated that it was the first report of protection afforded by a formalin inactivated, sonicated, adjuvant-free vaccine after challenge by BHV-1 and P. haemolytica.

Cardella et al. (1987) reported very little protection against P. haemolytica challenge in calves vaccinated intramuscularly with P. haemolytica - P. multocida bacterins adsorbed in aluminum hydroxide, while calves vaccinated with a bacterin incorporated into a mineral oil adjuvant had a high degree of protection after challenge.

Vaccination of calves with formalin-killed P. haemolytica in Freund's complete or incomplete adjuvant was associated with protection against experimental pneumonic pasteurellosis, whereas vaccination of calves with formalin-killed P. haemolytica in an aluminum hydroxide gel adjuvant did not protect calves against challenge (Confer et al., 1987).

Two studies of vaccination of calves with P. haemolytica bacterins have reported detrimental effects after challenge with P. haemolytica. Friend et al. (1977) reported that after challenge of calves vaccinated with a formalin-killed bacterin, serum antibody titers (direct bacterial whole cell agglutination, indirect bacterial agglutination, and passive hemagglutination) were positively correlated with the degree of pneumonic change, while nonvaccinated calves had antibody

titers that were negatively correlated with lung lesions. In this study, formalin-killed bacteria were administered in two doses, the first with Freund's complete adjuvant, the latter with Freund's incomplete adjuvant. Calves were also aerosol-exposed to formalin-killed P. haemolytica.

Wilkie et al. (1980) demonstrated increased severity and rate of pneumonia in bacterin-vaccinated calves that were subsequently challenged with P. haemolytica. Calves in this study were vaccinated subcutaneously one time with formalin-treated P. haemolytica emulsified in Freund's complete adjuvant. Subsequent serum antibody responses were generally low, but no correlations of antibody responses to lung lesions were made. The proposed reason for increased severity of pneumonia in calves in this study was that subcutaneous vaccination promoted production of opsonizing antibodies to P. haemolytica. Opsonizing antibodies to P. haemolytica had previously been associated with enhancement of P. haemolytica-induced macrophage cytotoxicity (Wilkie & Markham,1979), presumably resulting in increased phagocytosis of organisms. Conversely, Newman et al. (1982) associated increased resistance to pneumonic pasteurellosis with greater uptake and degradation of bacteria by pulmonary phagocytes; greater uptake and degradation of P. haemolytica should be dependent on opsonizing antibodies, which refutes the claim made by Wilkie et al. (1980) and Wilkie and Markham (1979).

The susceptibility of pulmonary phagocytes may be more dependent on the concentration of leukotoxin in the extracellular environment than on the degree of phagocyte uptake of P. haemolytica. Leukotoxin has been demonstrated on the plasma membranes of bovine alveolar macrophages in pneumonic lungs of calves infected with P. haemolytica (Whiteley et al., 1990), providing evidence that P. haemolytica may not need to be phagocytosed prior to mediating leukotoxin-induced phagocyte injury, if sufficient leukotoxin was present in the extracellular environment.

In summary of the protective effects of vaccination of calves with P. haemolytica bacterins, disparate responses have been demonstrated, ranging from beneficial to detrimental effects. There also appears to be no consistently beneficial effect when adjuvants were used with the P. haemolytica bacterins.

Live vaccines   Numerous studies have determined that subcutaneous vaccination of calves with live P. haemolytica vaccines induces protection against challenge (Newman et al., 1982, Kucera et al., 1983, Panciera et al., 1984, Catt et al., 1985, Corstvet et al., 1978, Confer et al. 1985a, Confer et al. 1986b). In one study, however, aerosol vaccination of calves with live P. haemolytica did not result in protection against pneumonia after challenge with P. haemolytica (Jericho and Langford, 1982).

Live vaccines may protect better than bacterins, at least in part because of induction of anti-leukotoxin antibodies by live vaccines (Wilkie et al., 1982). Since leukotoxin is produced only by actively growing P. haemolytica (Shewen and Wilkie, 1985), sufficient in vivo replication of organisms in live vaccines may be necessary to induce a protective level of anti-leukotoxin antibodies (Confer et al., 1986b). Alternatively, since leukotoxin is elaborated into supernatants of P. haemolytica cultures (Conlon et al., 1991), cell-free vaccines containing culture supernatant may serve to induce a protective level of anti-leukotoxin antibodies.

Component vaccines and protective antigens of Pasteurella haemolytica   Although live P. haemolytica vaccines are generally associated with protection against pneumonic pasteurellosis that is superior to that associated with P. haemolytica bacterins, undesirable responses indicative of P. haemolytica bacteremia and septicemia have been associated with vaccination of cattle with live P. haemolytica vaccines (Zeman et al.,1990). Purification of antigenic components of P. haemolytica that are associated with protection against pneumonic pasteurellosis would allow for effective immunization without the hazards of inoculation of potentially virulent live organisms. Several components of P. haemolytica have been evaluated as immunizing agents

against pneumonic pasteurellosis, and cell-free vaccines have been commercially licensed.

Matsumoto et al. (1984) vaccinated calves with a saline extract of P. haemolytica. Calves vaccinated subcutaneously with the extract in aluminum hydroxide gel had only mild pneumonia after challenge with P. haemolytica, while control calves had moderate to severe pneumonia. The nature of the extracted material was not identified, but McKinney et al. (1985) identified more than 15 proteins in a saline extract of P. haemolytica, and suggested that the proteins could be from outer membranes of the cell wall or inner membrane, or internal proteins due to cell rupture. Confer et al. (1985b) vaccinated calves with a saline extract of P. haemolytica in Freund's incomplete adjuvant, demonstrating protection by this method. After the challenge with P. haemolytica, the vaccinated calves had a mean lesion score of 2.0, versus 13.8 in control calves. The authors of this study suggested that the protective antigens in the extract may have been polysaccharide antigens from the capsule of the organism.

In two studies, potassium thiocyanate (KSCN) was used to extract glycoproteins from P. haemolytica, and the extracted glycoproteins were used as immunizing agents. Smith et al. (1983) determined that calves vaccinated with a KSCN extract developed serum antibodies to antigens in the extract. Yates et al. (1983) used a KSCN extract to vaccinate calves; 2/22

of the calves vaccinated subcutaneously or by aerosolization died after challenge with P. haemolytica, while 7 of 14 nonvaccinated control calves died after the challenge.

A sodium salicylate extract of P. haemolytica was used to vaccinate calves in a study by Gilmour et al. (1987); vaccinated calves had more severe clinical responses and pneumonia than nonvaccinated calves after challenge with P. haemolytica.

Confer et al. (1989) demonstrated protection of calves against experimental challenge by vaccination with a carbohydrate-protein extract of P. haemolytica. The extract was stated to be expected to contain capsular carbohydrates, outer membrane proteins, and low concentrations of LPS. High antibody responses to capsular carbohydrates were significantly correlated with low lung lesion scores after experimental challenge with P. haemolytica. In a similar study, Townsend et al. (1987) demonstrated seroconversion to capsular carbohydrate of P. haemolytica in calves vaccinated with live P. haemolytica or culture supernatants of P. haemolytica, although the calves were not experimentally challenged in efforts to correlate the serum antibody response with protection against disease.

Morton et al. (1990) reported that a sodium lauroyl sarcosinate insoluble preparation of P. haemolytica, containing outer membrane proteins, was effective in

protecting calves against the transthoracic challenge with P. haemolytica. Calves in this study were vaccinated twice with the outer membrane protein preparation combined with Freund's incomplete adjuvant.

Several studies have associated high anti-leukotoxin antibodies with resistance to pneumonic pasteurellosis (Shewen and Wilkie, 1983b, Gentry et al., 1985, Confer et al., 1986b, Shewen and Wilkie, 1988). However, calves immunized with recombinant leukotoxin of P. haemolytica A1 were afforded no protective advantage to challenge with virulent P. haemolytica (Conlon et al., 1991). In this study, however, calves were protected against experimental challenge after vaccination with a commercial P. haemolytica vaccine enhanced with recombinant leukotoxin. The commercial vaccine used was a P. haemolytica culture supernatant vaccine that confers anti-leukotoxin immunity and immune responses to other soluble antigens present in the culture supernatant (Shewen et al., 1988). In the study by Conlon et al. (1991), however, calves vaccinated with the commercial vaccine alone had a lower, but not significantly lower degree of pneumonia than saline-treated controls. The authors of this study suggested, like Confer et al. (1987) and Shewen and Wilkie (1988) that although neutralizing antibodies to leukotoxin are very important in protection against pneumonic pasteurellosis, antigens related to the bacterial surface are

probably necessary to induce protective immunity.

Cell-mediated immunity to Pasteurella haemolytica

Studies of bovine cell-mediated immunity (CMI) to P. haemolytica have not been published. Maheswaran and Thies (1979) demonstrated CMI to P. multocida in calves, using a whole blood lymphocyte stimulation assay. Calves were vaccinated with P. multocida bacterins containing Freund's incomplete adjuvant, then venous blood cultures, prepared two to three weeks after vaccination, were stimulated with sonicated fractions of P. multocida. Stimulation indices of up to 5.2 were reported in vaccinated calves, however indices in nonvaccinated calves were not reported.

Although certain humoral immune responses have been correlated with protection against pneumonic pasteurellosis (Confer et al., 1985a), CMI was not measured in the same study. It is possible that CMI was also contributing a major component to immune protection against P. haemolytica. Confer et al. (1985a) reported that calves vaccinated with a live vaccine were protected from challenge with P. haemolytica, while those vaccinated with a bacterin were not protected, even though antibody responses were not significantly different between the two groups. The authors suggested that a possible reason for the difference in protection coincident with no difference in antibody response may have been due to cell-mediated immune responses in the

calves vaccinated with a live vaccine.

Wells et al. (1979) compared lymphocyte proliferative responses to P. haemolytica antigens in a group of passively immunized lambs and a group of lambs vaccinated with an aluminum hydroxide adsorbed sodium salicylate extract of P. haemolytica. Lymphocyte proliferative responses were present only in actively immunized lambs, which were protected against subsequent challenge with P. haemolytica, while passively immunized lambs were not protected. This study provided evidence that CMI may be more important than humoral immunity in protection against pneumonic pasteurellosis.

Although lymphocyte proliferative assays are commonly used to evaluate CMI responses (Schultz, 1982), in vitro measurement of CMI responses may also be accomplished by measurement of specific cytokines that are elaborated in antigen-specific T lymphocyte responses, such as gamma interferon. Gamma interferon is produced only by antigen activated T lymphocytes (DeMaeyer and DeMaeyer-Guignard, 1992).

As an effector cytokine of the CMI response, gamma interferon may provide additional assistance in elimination of P. haemolytica by enhancing phagocyte uptake and killing of organisms. Gamma interferon has been shown to enhance cellular antimicrobial activities against several bacterial organisms, including Mycobacterium bovis (Flesch and

Kaufmann, 1988) and <u>Listeria</u> <u>monocytogenes</u> (Portnoy et al., 1989). Administration of gamma-interferon to mice has been shown to reduce infection rates after challenge with <u>Brucella</u> <u>abortus</u> (Stevens et al., 1992) and administration of gamma interferon to dexamethasone-immunosuppressed calves prior to challenge with <u>Haemophilus</u> <u>somnus</u> reduced the subsequent severity of pneumonia (Chiang et al., 1990)

Mice that were aerosol-infected with <u>Bordetella</u> <u>pertussis</u> developed in vitro lymphocyte proliferative responses to <u>B</u>. <u>pertussis</u> antigens at a simultaneous time period as in vitro gamma interferon responses. The time period of induction of these CMI responses coincided with clearance of <u>B</u>. <u>pertussis</u> from the lung, whereas induction of antibody responses occurred only when the infection was almost cleared. The authors of the study concluded that the CMI response was more important in clearance of organisms than the humoral response (Petersen et al., 1992). Although <u>B</u>. <u>pertussis</u> is generally considered to exist in the extracellular environment, it has been demonstrated to survive intracellularly in macrophages (Saukkonenet et al., 1991).

The potential beneficial effects of CMI against <u>P</u>. <u>haemolytica</u> are unknown. <u>Pasteurella</u> <u>haemolytica</u> survives and replicates in the extracellular environment (Confer et al., 1988), while studies of CMI responses have generally

involved intracellular organisms. Cell-mediated immune responses, however, would be expected to aid humoral immune responses in bactericidal activities against P. haemolytica, especially during and after phagocyte uptake of the organism.

PAPER 1. COMPARISON OF INTRATRACHEAL, INTRAVENOUS,
          AND INTRATONSILLAR ROUTES OF INOCULATION
          OF GOATS WITH PASTEURELLA HAEMOLYTICA

Comparison of intratracheal, intravenous, and intratonsillar routes of inoculation of goats with <u>Pasteurella haemolytica</u>

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INTRODUCTION

Factors involved in induction of bovine pneumonic pasteurellosis are poorly defined, and production of pneumonia by intratracheal inoculation of the causative agent, <u>Pasteurella</u> <u>haemolytica</u> is inconsistent (Yates, 1982). The organism is thought to gain access to the lung via the airways, originating from the nasopharynx which has been heavily colonized (Grey and Thompson, 1971). However, there are no reports of reproduction of the disease after inoculation of <u>P</u>. <u>haemolytica</u> directly into the nasal cavity of normal cattle.

Pneumonic pasteurellosis can be induced by intravenous inoculation of <u>P</u>. <u>haemolytica</u> biotype A serotype 1 (A1) (Thomas et al., 1989). Calves inoculated intravenously with this biotype developed pneumonic lesions with distribution patterns and gross and microscopic lesions very similar to those seen in natural cases of the disease. Also, <u>P</u>. <u>haemolytica</u> has been isolated from livers, spleens and mesenteric lymph nodes of cattle inoculated intrabronchially with <u>P</u>. <u>haemolytica</u>, suggesting that bacteremia had occurred (Anderson et al., 1991).

Goats are susceptible to pneumonic pasteurellosis under natural conditions (Young and Griffith, 1985). <u>Pasteurella</u> <u>haemolytica</u> has been isolated from the nasal cavity of goats, increased populations being associated with stressful

conditions (Jasni et al., 1991), as in cattle (Frank and Smith, 1983). This suggests that the goat can serve as a model for bovine pneumonic pasteurellosis.

Tonsils often serve as a reservoir for infectious agents, and lymphoid tissues are a site of entry for a number of pathogens (Woode et al., 1984, Ackermann et al., 1988, Momotani et al., 1988). Pasteurella haemolytica biotype A has been isolated from the tonsils of cattle and sheep (Al-Sultan and Aitken, 1985, Frank and Briggs, 1991), but it is not known whether it may invade the tonsils of ruminants and produce systemic disease.

The objectives of this study were to evaluate intratracheal, intravenous and intratonsillar routes of inoculation of P. haemolytica A1 in goats, and to characterize any lesions that developed after inoculation.

MATERIALS AND METHODS[1]

Animals

Ten colostrum-deprived, crossbred goats were reared in isolation until they were 14-18 weeks of age. Antibody levels to P. haemolytica, as determined by indirect hemagglutination (Frank and Smith, 1983), ranged from 1:4 to 1:16 in 7 goats. Pasteurella haemolytica was not isolated from nasal swabs prior to inoculation. Goats were randomly assigned to 3 treatment groups (3 goats per group) with one remaining as a sham inoculated control. Goats were inoculated with P. haemolytica A1 (isolate L101) that was isolated from pneumonic bovine lung. Goats inoculated intratracheally and intravenously were given 10 ml of an inoculum containing 2.0×10^5 CFU, while those inoculated intratonsillarly were given 0.1 ml of an inoculum containing 2.3×10^7 CFU, which was placed into the palatine tonsillar sinus. The control goat was inoculated with 10 ml of sterile tryptose broth intratracheally and intravenously, and 0.1 ml intratonsillarly.

Goats were killed and necropsied 42-66 hours after inoculation and tissue samples were collected for quantitative bacterial culture and for histopathology.

[1]A more detailed description of materials and methods is presented in Appendix 3.

RESULTS

All goats inoculated intratracheally had pulmonary lesions which consisted of consolidation of the ventral portions of the anterior and middle lobes. One of these 3 goats had a 5 cm raised focus of consolidation and hemorrhage in the caudal lobe that was overlain with pleural fibrin deposits. Approximately 11, 15, and 22% of the total lung volume was consolidated in each of these goats.

One of the goats inoculated intravenously had gross pulmonary lesions. Anterior lobes and the middle lobe of this goat had consolidation of the ventral portions, in addition to a 6 cm diameter focus in the right caudal lobe. Approximately 17 % of the lung volume of this goat was consolidated. A thick fibrinous exudate covered the middle lung lobe, and extended ventrally over the mediastinal surface.

No gross lesions were present in the lungs or tonsils of the goats that were inoculated intratonsillarly, nor were significant gross lesions present in other tissues of any goats. No gross lesions were present in the sham-inoculated goat.

Histopathological findings

The lung lesions in the transtracheally inoculated goats consisted of extensive areas of fibrinosuppurative to necrotizing bronchopneumonia. The alveolar spaces were

filled with serofibrinous fluid and leukocytes, primarily neutrophils with fewer macrophages. Multifocally, bronchioles contained similar exudates, and interlobular septa were widened by deposition of fibrin and edema. Alveolar septa had increased numbers of type II pneumocytes. Subpleural hemorrhage and infiltrates of low numbers of neutrophils into the pleura were multifocally present.

The lung lesions in the intravenously inoculated goat were similar to those in the transtracheally inoculated animals. Differences consisted of increased type II pneumocyte hyperplasia and increased severity of fibrinous pleuritis in the intravenously inoculated goat. There were areas where a layer of fibrin up to 50 micrometers thick was present on the pleural surface of the lung. Pleuritis in the intravenously inoculated goat was often seen over unconsolidated lung parenchyma, but pleuritis in the transtracheally inoculated goats was typically over consolidated lung.

No microscopic lesions were present in the lungs from the intratonsillarly inoculated goats, or in other tissues from inoculated goats. There were no microscopic lesions present in tissues from the sham inoculated control goat.

Microbiological findings

Of the goats inoculated intravenously, P. haemolytica was isolated from blood samples taken at 1 min after

inoculation (330, 240, and 180 CFU/ml respectively). No bacteria were isolated from other blood samples.

Pasteurella haemolytica A1 was isolated from the tonsils of all 3 intratonsillarly inoculated goats at 7 X 10^3, 2 X 10^5, and 5 X 10^6 CFU/g of tonsil, respectively. Four X 10^3 CFU/g of tissue were isolated from a lung lesion of one goat inoculated transtracheally. Pasteurella spp. were not isolated from the lungs of other goats, nor from any other tissues of remaining goats.

DISCUSSION

Inoculation of goats by the transtracheal route resulted in pneumonia in 3 of 3 goats, indicating that the goat may serve as a suitable model for studies of bovine pneumonic pasteurellosis.

As P. haemolytica A1 was isolated from blood only immediately after intravenous inoculation, bacteria were apparently rapidly cleared by fixed tissue macrophages or rapidly killed or altered by serum-associated bacteriolytic factors.

The lack of isolation of P. haemolytica from the pneumonic lung in 3 of the 4 goats in this study, may have been due to effective host responses in eliminating viable organisms by the time of necropsy. The body temperatures had declined by then, also suggesting a reduction in the inflammatory processes associated with the elimination of viable P. haemolytica from the pneumonic foci.

Intravenous inoculation of goats in the present study resulted in less consistent production of pneumonia and a lesser amount of pulmonary involvement than in the study by Thomas et al. (1989), in which 5 of 5 calves had pneumonic lesions with 14% to 46% (mean 28%) of the pulmonary parenchyma being consolidated after intravenous inoculation of calves with approximately 1 X 10^8 CFU of P. haemolytica A1. The lesser rate and degree of pneumonia in the present

study may have been associated with the lower number of organisms used. In the study by Thomas et al. (1989), the organisms were apparently inoculated with accompanying culture supernatant, and calves may have been given large amounts of endotoxin, leukotoxin, and/or other bacterial components that could alter normal host defenses against blood borne pathogens.

There was no evidence of invasion of P. haemolytica A1 via the palatine tonsil after inoculation, even though 3 of 3 goats inoculated intratonsillarly had tonsils colonized by the organism. This suggests that this isolate of P. haemolytica failed to invade, or, if invasion occurred, intravascular phagocytosis eliminated organisms without formation of lesions. Other host factors such as environmental stress may enhance invasion by certain bacteria, and such stressors or similar factors may have been lacking in this study.

The establishment of pneumonic lesions after intravenous inoculation of bacterial organisms implies that large numbers of bacteria are deposited in the lung. Ruminant lungs possess pulmonary intravascular macrophages which are responsible for the clearance of most intravascular bacteria, whereas some species of animals (such as the rat) that lack pulmonary intravascular macrophages have clearance of intravascular organisms primarily in the spleen and liver

(Warner et al., 1987). Therefore, the lung may be a target of deposition of bacteria following intravenous inoculation in ruminants. However, Thomas et al. (1989) state that inoculation of other Gram-negative bacteria into cattle does not result in replication of bacteria in the lung as does inoculation with P. haemolytica, indicating that there are other factors involved in predilection of P. haemolytica to pulmonary tissue in cattle.

The low antibody titers to P. haemolytica in the goats indicates probable exposure to the organism even though they were raised in isolation. How low levels of antibody may have affected the outcome of this study is unknown, although it has been shown that calves vaccinated with P. haemolytica bacterins may have more severe disease than non-vaccinates (Wilkie et al., 1980). Of interest is the fact that the goat in this study with the most lung consolidation also had the highest antibody titer to P. haemolytica. No other correlations between serum antibody level and degree of pneumonia could be drawn.

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PAPER 2. IN VITRO LYMPHOCYTE PROLIFERATIVE RESPONSES
AND GAMMA INTERFERON PRODUCTION AS MEASURES
OF CELL-MEDIATED IMMUNITY OF CATTLE EXPOSED
TO PASTEURELLA HAEMOLYTICA

In vitro lymphocyte proliferative responses and gamma interferon production as measures of cell-mediated immunity of cattle exposed to <u>Pasteurella haemolytica</u>

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ABSTRACT

Cell-mediated immune mechanisms may play a role in the pathogenesis and prevention of pneumonia in cattle that is caused by <u>Pasteurella haemolytica</u> serotype A1. The circumstances required to stimulate and identify cell-mediated immune mechanisms were determined in calves. Calves were vaccinated with a commercial <u>P. haemolytica</u> bacterin or a live commercial <u>P. haemolytica</u> vaccine, or were infected intratracheally with virulent <u>P. haemolytica</u>. All calves were challenge-exposed intratracheally with <u>P. haemolytica</u> 31 days after vaccination or infection. Peripheral blood mononuclear cells and mediastinal and superficial cervical lymph node cells were stimulated with antigens prepared from <u>P. haemolytica</u> to evaluate in vitro proliferative responses and gamma interferon production as measures of cell-mediated immunity. Strong proliferative responses and gamma interferon production were detected in lymph node cells from calves vaccinated with the live vaccine and infected calves, in response to stimulation with an outer membrane protein preparation from <u>P. haemolytica</u>. Greater proliferative responses and gamma interferon production were associated with the lymph node nearer the site of bacterin administration (superficial cervical lymph node) or the site of infection (mediastinal lymph node), whereas greater proliferative responses and gamma interferon production were

associated with the more distant lymph node (mediastinal lymph node) in calves vaccinated with the live vaccine. Neither proliferative responses nor gamma interferon production were detected in peripheral blood mononuclear cells from calves that were vaccinated for or infected with P. haemolytica. Protection against pneumonic lesions was more closely correlated with antibody responses than with proliferative or gamma interferon responses.

INTRODUCTION

Pasteurella haemolytica biotype A serotype 1 causes severe pneumonia in cattle. Numerous experimental and commercial vaccines, including killed bacterins and live vaccines have been developed for immunization of cattle against P. haemolytica. Generally, immunization with P. haemolytica bacterins provides poor protection of cattle against natural or experimental disease (2, 20, 21) and has even been associated with enhancement of experimental and natural disease (16, 33). Immunization of calves with bacterins in Freund's adjuvant (7), or live P. haemolytica (5, 6, 10, 25), however, has been demonstrated to provide protection against challenge.

Pasteurella haemolytica produces a potent leukotoxin that is inhibitory or lethal to bovine neutrophils, mononuclear phagocytes, and lymphocytes (11, 12, 22). Investigators have attributed the protection afforded by live vaccines to their induction of leukotoxin-neutralizing antibodies (18). Calves protected against P. haemolytica challenge by immunization with a bacterin in Freund's adjuvant, however, did not produce leukotoxin-neutralizing antibodies, indicating that antibodies to components other than leukotoxin can afford protection (7). Subsequently, it was demonstrated that immunization of calves with recombinant bovine leukotoxin did not result in protective immunity (9),

indicating that effective humoral immune protection probably
requires antibody responses to somatic antigens of P.
haemolytica in addition to anti-leukotoxin antibodies.

Humoral immune mechanisms are thought to be important in
the defense against pneumonic pasteurellosis. Pasteurella
haemolytica is killed after incubation with antiserum as a
result of complement-mediated killing (23), and opsonization
of P. haemolytica enhances phagocyte ingestion of the
organism (24). However, protection against pneumonic
pasteurellosis does not always correlate with antibody
titers. Confer et al. (60) immunized cattle with killed or
live P. haemolytica, and both groups developed similar serum
antibody titers. The cattle immunized with live organisms
were more resistant to challenge with virulent organisms than
those immunized with killed organisms, leading the authors to
suggest that the enhanced resistance may have resulted from
the ability of live organisms to stimulate stronger cell-
mediated immune (CMI) responses than killed organisms, even
though antibody responses were not significantly different.

There is additional evidence that CMI may be important
in protection against pneumonic pasteurellosis. Wells, et
al. (32) demonstrated that lambs that were passively
immunized with P. haemolytica antiserum were not protected
from disease after challenge with P. haemolytica, whereas
lambs immunized with a sodium salicylate extract were

resistant to challenge, even though antibody titers were not significantly different between the two groups. A CMI response was detected only in the lambs that were actively immunized, which may have been an important protective factor against the P. haemolytica challenge.

The purpose of this study was to identify CMI responses of cattle to P. haemolytica, as well as identify P. haemolytica antigens that can be used to identify this response. Calves infected with P. haemolytica and calves vaccinated with a live or killed P. haemolytica vaccine were used to evaluate CMI responses to P. haemolytica. Peripheral blood mononuclear cells (PBMC) and lymph node cells were harvested and stimulated with 5 antigen preparations from P. haemolytica. Lymphocyte proliferative responses and gamma interferon production were used as measures of CMI.

To assess the role of CMI versus humoral immunity as protective factors against pneumonic pasteurellosis, all calves were challenge-exposed to P. haemolytica, and lung lesions and bacterial concentrations in lung tissues were assessed for correlation with CMI and antibody responses.

MATERIALS AND METHODS

Bacterial culture

Pasteurella haemolytica biotype A serotype 1 (strain
L101, kindly provided by G. H. Frank, Ames, IA) was isolated
on blood agar from a pneumonic bovine lung. Eight isolated
colonies were inoculated into tryptose broth and incubated at
37 C for 6 hours, aliquoted into vials, and stored at -70 C
until used for antigen preparations or inoculations.

Antigen preparation

Capsular polysaccharide (CP), lipopolysaccharide (LPS),
lipopolysaccharide-associated protein (LAP), and a bacterial
cell sonicate (SON) were prepared from P. haemolytica strain
L101 to use as antigens to stimulate PBMC and lymph node cell
cultures. An outer membrane protein preparation (OMP) of P.
haemolytica serotype 1 was kindly provided by A. W. Confer,
Stillwater, OK, prepared as previously described (28).

Strain L101 was grown on dextrose starch agar for 24
hours at 37 C, and harvested in distilled water. Bacteria
were pelleted by centrifugation and the supernatant was
saved. Cells were dehydrated in 50% ethanol, washed twice in
acetone, twice in ethyl ether, and then air-dried.

The supernatant was placed in dialysis bags (12-14,500
nominal mol. wt. cutoff, Spectrum Medical Industries, Inc.,
Los Angeles, CA) and covered for 18 hours with
carboxymethylcellulose (Aquacide I, Calbiochem Corp, La

Jolla, CA). The CP was extracted from the concentrated

culture supernatant as previously described (1). Alpha-

amylase, (Sigma Chemical Co., St Louis, MO) 500 U/ml, was

added and the mixture was incubated for 2 hours at 37 C and

18 hours at 4 C to remove residual starch derived from the

growth medium. The CP was precipitated with ethanol and

sodium acetate, and resuspended in a buffer (pH 7.2)

containing 10 mM Tris, 145 mM NaCl, 0.2% SDS and 500 ug (16

U)/ml proteinase K (Amresco, Solon, OH). The suspension was

heated at 60 C for 2 hours then 18 hours at 37 C. The

suspension was recycled for 1 hour over an endotoxin-removing

affinity column (Detoxi-gel, Pierce, Rockford, IL). The CP

was again precipitated with ethanol and sodium acetate,

resuspended in pyrogen-free distilled water (Baxter

Healthcare Corp, Deerfield, IL), precipitated and resuspended

again, and lyophilized. The CP, which contained less than

0.25% protein and 0.01 % LPS, was reconstituted in Hanks

balanced salt solution (HBSS) (Gibco Laboratories, Long

Island, NY) and stored at -20 C.

Lipopolysaccharide was extracted from dried cells with a

phenol, chloroform, and petroleum ether solution, as

previously described (17). The LPS was resuspended in

distilled water, heated at 56 C for 30 min, lyophylized,

resuspended in a buffer (pH 7.2) containing 10 mM Tris, 145

mM NaCl, 0.2% SDS and 500 ug (16U)/ml proteinase K. The

suspension was heated for 2 hours at 60 C, then was incubated
for 18 hours at 37 C. The LPS was precipitated from the
buffer with ethanol and sodium acetate, and the precipitate
was resuspended in pyrogen-free distilled water. The LPS was
precipitated again in ethyl alcohol and sodium acetate,
resuspended in pyrogen-free distilled water, and lyophilized.
The LPS, which contained approximately 0.7% protein (3), was
resuspended in HBSS at 0.4 mg/ml and stored at -20 C.

Lipopolysaccharide-associated protein was extracted from
the phenol supernatant of the LPS extraction as previously
described (30). Extracted LAP was washed 3 times with
ethanol, 3 times with ethyl ether, dried, reconstituted in
pyrogen-free distilled water, and lyophilized. The LAP
contained 0.02% LPS as determined by a chromogenic limulus
amebocyte assay (QCL-1000, Whittaker Bioproducts,
Walkersville, MD).

A bacterial cell sonicate was prepared by collecting P.
haemolytica cells from 18 hours of growth on sheep blood agar
plates that were incubated at 37 C. Cells were collected in
distilled water and sonicated on ice with an ultrasonic cell
disrupter (Microson, Heat Systems Ultrasonics, Farmingdale,
N.Y.) for 1 min at the maximum power setting. The
preparation was centrifuged at 5900 X g for 20 min., and the
supernatant was filtered through a 0.22 micrometer filter,
lyophilized, reconstituted to 0.4 mg/ml in HBSS, and stored

at -20C.

Calves

Twelve 3-7 month-old, colostrum-deprived, male Holstein and Holstein crossbred calves were housed in isolation barns in American Association for Accreditation of Laboratory Animal Care (AAALAC) accredited facilities. Calves were fed a complete pelleted ration twice daily, and water was available ad libitum.

Vaccination and infection of calves

Calves were divided into 4 groups of 3 calves, and were vaccinated or infected with P. haemolytica on day 0. Calves of one group (bacterin group) were vaccinated with a Pasteurella haemolytica-Pasteurella multocida bacterin (Pasteurella haemolytica-Pasteurella multocida bacterin, Lot No. 923, Colorado Serum Co., Denver, CO). Calves in a second group (live vaccine group) were vaccinated with a live P. haemolytica vaccine (Respirvac, Lot No. 7671, Beecham Laboratories, Bristol, TN). Vaccines were administered in the subcutis of the left cervical area. Calves in a third group (convalescent group) were intratracheally infected with 10 ml of an inoculum containing 3.0×10^8 CFU of P. haemolytica strain L101. The inoculum was prepared by inoculating 1 ml of aliquotted strain L101 into 30 ml of tryptose broth and incubating at 37 C for 3 hours. Bacteria were pelleted by centrifugation at 5900 X g for 5 min.,

resuspended in tryptose broth to 80% transmittance at 600 nm wavelength (Coleman Jr. Model 35 spectrophotometer, Bacharach Instrument Co., Pittsburgh, PA), then further diluted in tryptose broth to approximately 1 X 10^8 CFU/ml for inoculation of calves. Bacterial concentration was verified by plate counts. Calves in the convalescent group were treated with antibiotics (Gentocin, 2 mg/kg intramuscularly, Schering Corp., Kenilworth, N. J., and Naxcel, 2 mg/kg, intramuscularly, Upjohn Co., Kalamazoo, MI) on days 3-6 to reduce the morbidity following infection with P. haemolytica. A fourth group (unvaccinated group) consisted of calves that received no exposure to P. haemolytica. Body temperatures of all calves were monitored daily for 3 days after initial vaccination or infection. Calves in the bacterin group were re-vaccinated on day 15 of the experiment.

Antibody titers

Serum samples were collected from all calves on days 1, 4, 8, 11, 15, 18, 22, 25 and 31 for determination of antibody titers to P. haemolytica strain L101, by an indirect hemagglutination assay (15). Serum antibody titers were converted to $\log_2$ prior to statistical analyses, with a mean of 0.6 on day 0.

Lymphocyte proliferation assays

Blood was collected from calves on days 1, 4, 11, 15, 18, 22, and 25 for isolation of PBMC for lymphocyte

proliferation assays. Fifty ml of venous blood was collected into 5 ml of acid citrate dextrose, centrifuged at 600 X g for 25 min, and buffy coats were collected. Erythrocytes in buffy coats were lysed with phosphate buffered water, followed by restoration of osmolarity with hypertonic phosphate buffered saline. Peripheral blood mononuclear cells were washed twice in HBSS, and resuspended in complete medium composed of RPMI 1640 (Gibco Laboratories, Long Island, NY) supplemented with 25 mM L-glutamine, 5 X 10^{-5} M 2-mercaptoethanol, 200 IU penicillin/ml, 200 ug streptomycin/ml, 0.5 ug amphotericin B/ml, and 10% bovine fetal serum (Hy-Clone Laboratories, Logan, UT). Two-hundred ul of complete media containing 2 X 10^5 PBMC were delivered to each well of flat-bottom microtiter plates. Antigens (CP, LPS, LAP, and OMP), in 25 ul of RPMI, were added to wells in triplicate at concentrations of 1.0 and 0.1 ug/well. The SON antigen was added at 0.1 and 0.01 ug/well. Cell cultures were incubated for 52-54 hours at 37 C in a humidified atmosphere with 5% CO_2, and pulse-labelled with H^3-thymidine (Amersham Corp, Arlington Heights, IL) at 0.4 uCi/well. Cells were incubated an additional 18-20 hours, harvested onto fiberglass filters, and radioactivity was measured by liquid scintillation counting. Counts per minute (CPM) of background wells (no antigen) were subtracted from CPM of antigen-stimulated wells, and the difference was

designated difference in counts per minute (DCPM).

For lymph node cell assays, lymph nodes were minced and cells were passed through #60 mesh stainless steel screens. Lymphocytes were isolated using Histopaque 1083 (Sigma Chemical Co., St. Louis, MO), washed twice in 0.1 M phosphate-buffered saline, pH 7.2, and resuspended at 2×10^5 cells/ml in complete media. Lymph node cell preparations were thereafter treated in the same manner as PBMC preparations.

Gamma interferon assays

Peripheral blood mononuclear cell and lymph node cell suspensions were added to plates and stimulated with antigens in the identical manner as that used for the lymphocyte proliferation assays. After 46-48 hours incubation, plates were centrifuged at 100 X g for 5 min, and supernatants were collected and immediately frozen and stored at -20 C.

Gamma interferon was measured using a commercial ELISA kit (Mycobacterium paratuberculosis gamma-interferon test kit, IDEXX Laboratories, Westbrook, ME), with modifications. Culture supernatants were substituted for plasma samples, and recombinant gamma interferon (supplied by IDEXX Laboratories) was used as a standard for quantitative determinations. Supernatants from PBMC cultures from days 4, 11, and 18 that were stimulated with OMP at 0.1 ug/well and supernatants from lymph node cell cultures that were stimulated with OMP at 0.1

ug/well were analyzed for gamma interferon. Gamma interferon concentrations in supernatants of background wells were subtracted from those of supernatants of antigen-stimulated wells and the difference was designated difference in interferon (DIFN).

Challenge and necropsy

On day 31 of the experiment, all 12 calves were challenge-exposed intratracheally to 10 ml of inoculum containing 2.0 X 10^8 CFU of P. haemolytica. Body temperatures were monitored daily for 3 days after the challenge-exposure. Calves were euthanatized and necropsied on days 35 (6 calves) and 36 (5 calves). One calf from the unvaccinated group died on day 33 with severe pneumonic lesions as a result of the challenge.

The caudal mediastinal and superficial cervical lymph node (ipsilateral to site of vaccination) were removed at necropsy for lymph node cell preparations in the lymphocyte proliferation and gamma interferon assays.

The volume of acute pneumonic parenchyma was estimated for each calf and recorded as % pneumonia. Sections of lung were fixed in 10% neutral buffered formalin for histopathology. Lung sections were processed and embedded by routine paraffin techniques, sectioned, and stained with hematoxylin and eosin by standard methods.

A section of pneumonic lung was collected from each

animal for quantitative bacterial culture, and the CFU of P.
haemolytica/g of lung were converted to $\log_{10}$ prior to
statistical analyses.

Statistical analyses

Analysis of variance (SAS Institute Inc., Cary, N.C.)
was used to determine significant differences between group
means for DCPM, DIFN, antibody titer, body temperature, %
pneumonia, and CFU of P. haemolytica/g of lung. When the
overall F test was significant ($P \leq 0.05$), t tests (LSD) were
used to determine specific differences between group means
($P \leq 0.05$). Linear regression analysis of serum antibody titer
on day 31, DIFN and DCPM from mediastinal lymph node cells
stimulated with OMP at 0.1 ug/well versus % pneumonia and CFU
of P. haemolytica/g of lung were used to correlate immune
responses to the severity of pulmonary disease. Correlation
coefficients were also determined for DIFN versus DCPM (from
mediastinal lymph node cells stimulated with OMP at 0.1
ug/well) to correlate gamma interferon responses with
proliferative responses, and DCPM and DIFN from mediastinal
lymph node cells stimulated with OMP at 0.1 ug/well were
correlated with serum antibody response on day 31 to
determine relationships between CMI and humoral immune
responses.

RESULTS

### Clinical responses of calves

By 20 hr after vaccination or infection, calves in the live vaccine and convalescent group had significantly elevated body temperatures ($P \leq 0.05$) over calves in the unvaccinated group. Body temperatures in these two groups remained elevated until approximately 96 hours after vaccination or infection. All three calves in the live vaccine group developed extensive swelling at the site of vaccination, which extended ventrally to the sternum in one calf.

Calves in the convalescent group had clinical evidence of pneumonia after infection on day 0, which included hyperpnea, anorexia, and coughing. However, signs subsided by day 5.

Antibody responses

Serum antibody titers peaked for the live vaccine and convalescent group between days 8 and 11 (Fig. 1). Calves in the bacterin group, which were re-vaccinated on day 15, had a maximum mean antibody titer on day 22. On day 11, mean antibody titers of the bacterin, live vaccine and convalescent groups were significantly greater than that of the unvaccinated group ($P \leq 0.05$), while the mean titers of the live vaccine and convalescent groups were also greater than that of the bacterin group. There were no significant

Fig. 1. Mean serum antibody titers of calves by experimental
group as determined by an indirect hemagglutination
assay. Calves were vaccinated or infected with $\underline{P}$.
$\underline{\text{haemolytica}}$ on day 0, and calves in the bacterin
group were re-vaccinated on day 15. There is a
significant difference ($P \leq 0.05$) between group means
within a day when a common letter is not present.
Standard error of the means vary from 0.5 to 0.9
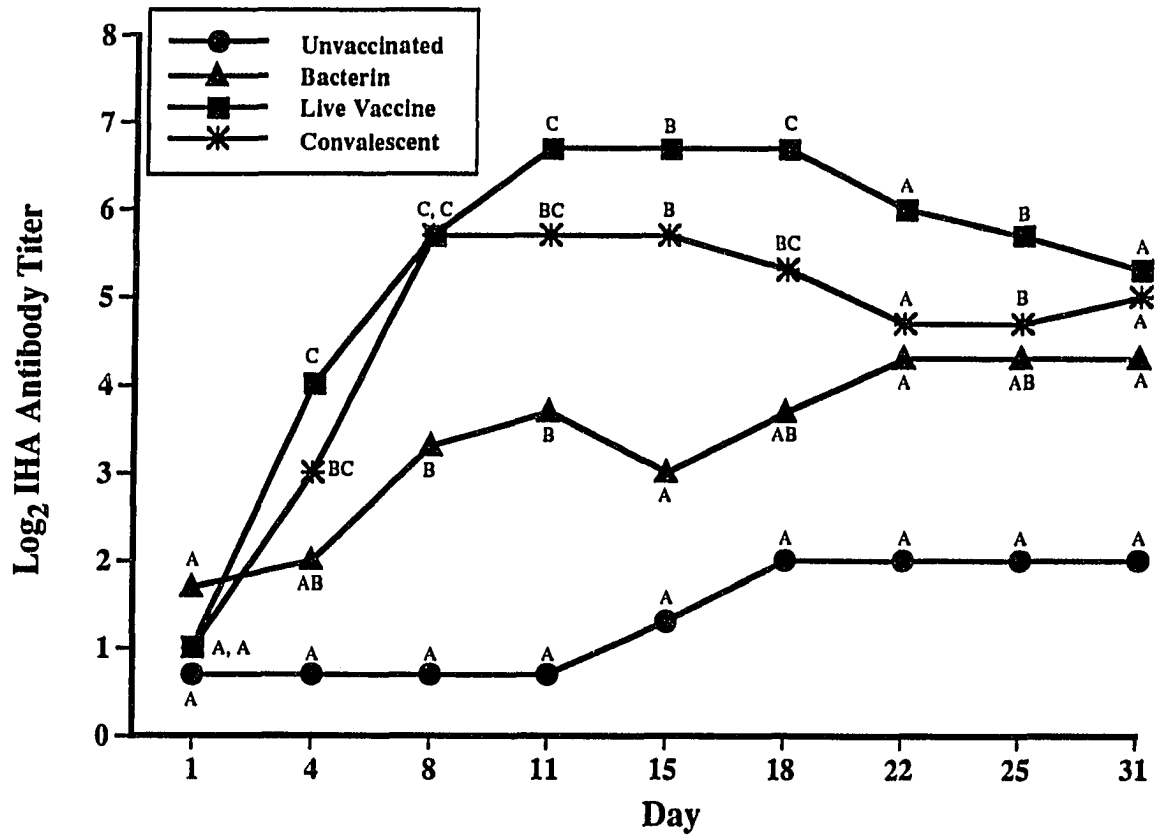
differences (P=0.07) between any of the group mean antibody titers on day 31.

Proliferative responses

Proliferative responses (DCPM) for PBMC and lymph node cells stimulated with CP, LPS, and LAP were unremarkable when compared to background wells, with DCPM generally below 2 X 10^3 CPM. Differences between experimental groups were not present in mean DCPM of PBMC or lymph node cells stimulated with CP, LPS, or LAP. Additionally, there were no significant experimental group differences for the mean DCPM of PBMC stimulated with OMP or SON antigens at any time period. The DCPM for PBMC stimulated with OMP or SON were also generally below 2 X 10^3 CPM (data not shown).

Lymph node cells stimulated with OMP at 0.1 ug/well had higher DCPM than those stimulated with OMP at 0.01 ug/well, and those stimulated with SON at 0.01 ug/well had higher DCPM than those stimulated with SON at 0.1 ug/well. Therefore, all reported responses are from cells stimulated with OMP at 0.1 ug/well and SON at 0.01 ug/well.

The DCPM of mediastinal lymph node cells from the convalescent group that were stimulated with OMP was significantly higher than the DCPM from mediastinal lymph node cells from the unvaccinated, bacterin, or live vaccine group when stimulated with OMP (P≤0.02) (Fig. 2). Significant differences between experimental groups were not

Fig. 2. Proliferative responses of superficial cervical and
mediastinal lymph node cells stimulated with outer
membrane protein (OMP) from P. haemolytica. Calves
were vaccinated or infected with P. haemolytica 35
or 36 days prior to stimulation of lymph node cells.
Values represent mean DCPM (difference of CPM of
antigen-stimulated wells and CPM of background
wells) by experimental group. There is a
significant difference ($P \leq 0.05$) between the
experimental group means within a lymph node when a
common letter is not present. Error bars indicate
SEM
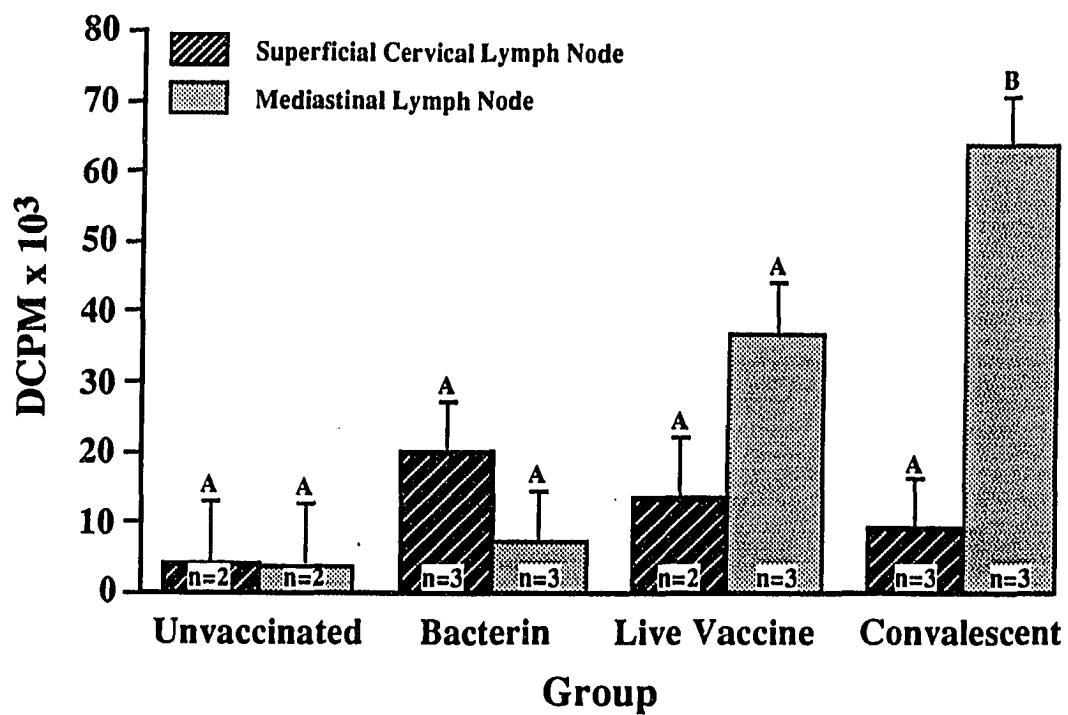
present in the DCPM of superficial cervical lymph node cells stimulated with OMP (P>0.05).

A significant difference in DCPM between superficial cervical and mediastinal lymph node cells within experimental groups was present in OMP-stimulated cells (P≤0.02) (Fig. 2). Calves in the bacterin group had a higher DCPM in superficial cervical than in mediastinal lymph node cells, while calves in the live vaccine and convalescent groups had a higher DCPM in mediastinal than in superficial cervical lymph node cells.

When the DCPM were averaged over superficial cervical and mediastinal lymph nodes cells stimulated with OMP, differences between experimental groups were present (P≤0.03) (Fig. 3). The live vaccine and convalescent group had a significantly higher DCPM than the unvaccinated group, while the DCPM of the convalescent group was also significantly higher than that of the bacterin group.

Differences between experimental groups were present in proliferative responses of mediastinal lymph node cells stimulated with SON (P<0.02), with the convalescent group having a significantly higher DCPM than the unvaccinated, bacterin, or live vaccine group (data not shown). A significant difference in DCPM between superficial cervical and mediastinal lymph node cells within experimental groups was present in SON-stimulated cells (P≤0.05). Calves in the bacterin group had a higher DCPM in superficial cervical than

Fig. 3. Proliferative and gamma interferon responses
averaged over superficial cervical and mediastinal
lymph node cells stimulated with outer membrane
protein (OMP) from P. haemolytica. Calves were
vaccinated or infected with P. haemolytica 35 or 36
days prior to stimulation of lymph node cells. The
values represent mean DCPM or DIFN (difference of
CPM or ng/ml of gamma interferon of antigen-
stimulated wells from background wells) by
experimental group, averaged over the superficial
cervical and mediastinal lymph nodes. There is a
significant difference ($P \leq 0.05$) between group means
within either DCPM or DIFN when a common letter is
not present. Error bars indicate SEM
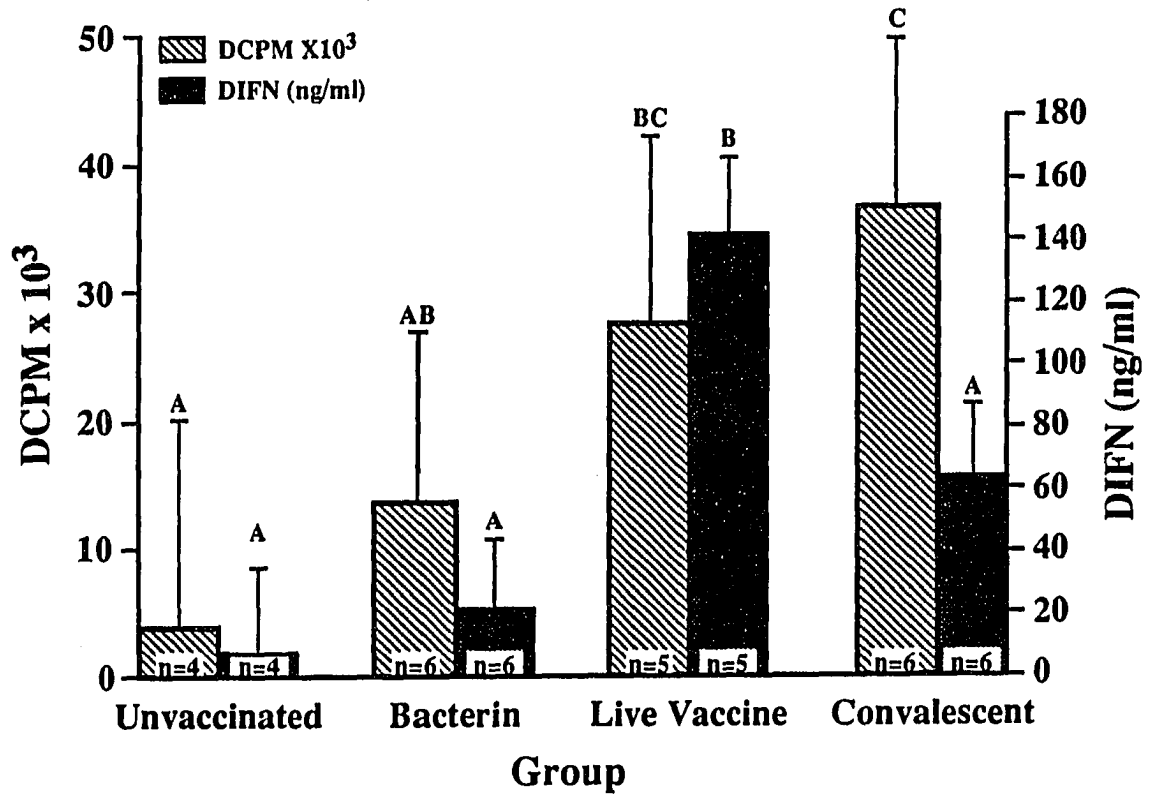
in mediastinal lymph node cells, while calves in the live

vaccine and convalescent groups had a higher DCPM in

mediastinal than in superfical cervical lymph node cells

(data not shown).

Differences between experimental groups were present in

proliferative responses averaged over superficial cervical

and mediastinal lymph node cells stimulated with SON

($P \leq 0.03$). The convalescent group DCPM was significantly

higher than the DCPM of the unvaccinated, bacterin, or live

vaccine group DCPM (data not shown).

Gamma interferon responses

Significant differences between experimental groups were

not present for DIFN from culture supernatants of PBMC

stimulated with OMP for any of the days analyzed (Days 11,

18, and 25) ($P > 0.05$) (data not shown).

Differences in gamma interferon production between

experimental groups were present from mediastinal lymph node

cells stimulated with OMP ($P \leq 0.002$) (Fig. 4). The highest

DIFN occurred in the live vaccine group, which was

significantly higher than the DIFN of the unvaccinated,

bacterin or convalescent group. The convalescent group DIFN

was also significantly higher than the DIFN of either the

unvaccinated or bacterin group.

Significant differences between experimental groups were

not present in the DIFN of superficial cervical lymph node

Fig. 4. Gamma interferon responses of superficial cervical

and mediastinal lymph node cells stimulated with

outer membrane protein (OMP) from P. haemolytica.

Calves were vaccinated or infected with P.

haemolytica 35 or 36 days prior to stimulation of

lymph node cells. Values represent mean DIFN

(difference of ng/ml of gamma interferon in antigen-

stimulated wells from background wells) by

experimental group. There is a significant

difference ($P \leq 0.05$) between group means

within a lymph node when a common letter is not

present. Error bars indicate SEM
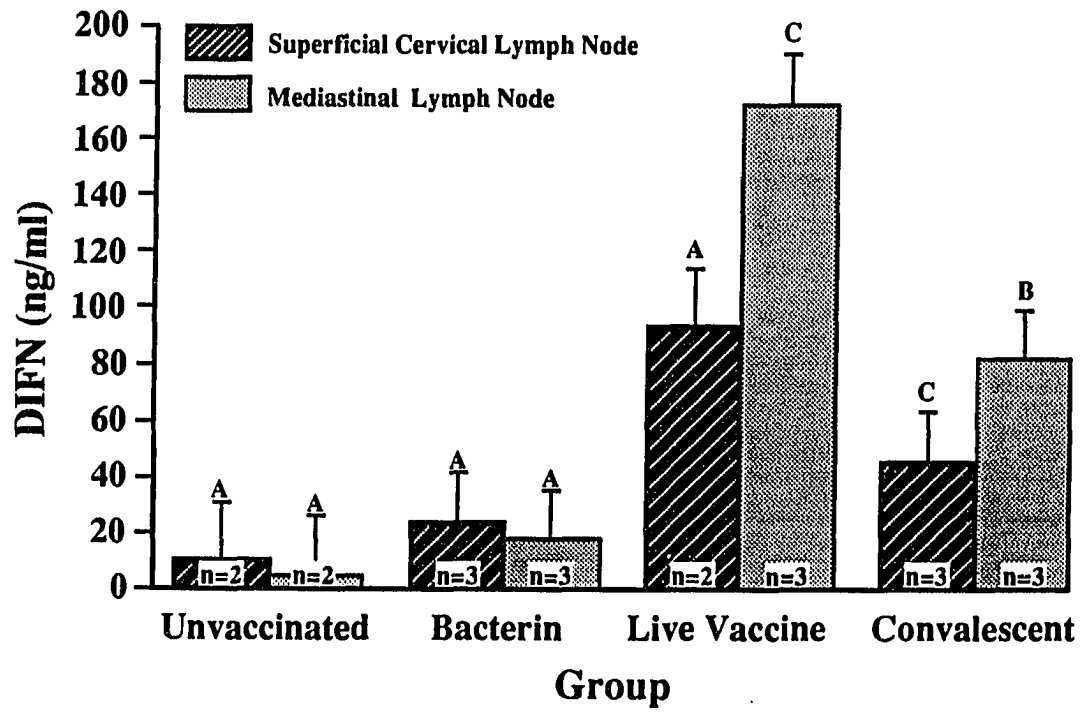
cells stimulated with OMP (P>0.05) (Fig. 4).

Differences between experimental groups were present in DIFN averaged over superficial cervical and mediastinal lymph node cells stimulated with OMP (P≤0.002) (Fig. 3). The live vaccine group average DIFN was significantly higher than the average DIFN of the unvaccinated, bacterin, or convalescent group.

Necropsy findings

Acute pneumonic lung consisted of consolidated parenchyma of ventral portions of the lobes, often accompanied by hemorrhage and fibrinous pleuritis. Microscopically, alveoli were filled with fibrin, neutrophils, macrophages, and ocassionally, streaming leukocytes. There were occasional foci of necrosis in the lung parenchyma, and interlobular septa and subpleural spaces were expanded by fibrinocellular exudate. The gross and microscopic lung lesions were consistent with previous descriptions of lesions associated with pneumonic pasteurellosis (19). The mean % pneumonia for each group was: unvaccinated - 25.1, bacterin - 10.5, live vaccine - 16.5, and convalescent - 0.1 (SEM=9.6). No signnificant differences occurred between experimental groups (P=0.37).

Chronic lung lesions were present in two calves of the convalescent group, characterized by abscessation of ventral portions of all lung lobes. These lesions were consistent

with pneumonia of several weeks duration, as a result of infection of the calves with P. haemolytica on day 0.

Bacterial cultures of lungs revealed pure populations of P. haemolytica. Mean CFU $\log_{10}$/g of lung by experimental group were: unvaccinated group - 4.9, bacterin group- 5.0, live vaccine group - 7.6, and convalescent group - 5.8 (SEM=1.6). There were no significant differences (P=0.62) between the experimental group means.

Correlations

Correlation of DCPM or DIFN of OMP-stimulated mediastinal lymph node cells with % pneumonia was not significant (P>0.05) (Fig 5). Correlation of DCPM or DIFN of OMP-stimulated mediastinal lymph node cells with bacterial concentration in pneumonic lesions was not significant (P>0.05) (data not shown). Serum antibody titer on day 31 and % pneumonia were significantly correlated, with r=-0.57 (P=0.05) (Fig. 5). However, this correlation was markedly affected by one seronegative calf in the unvaccinated group that had 58% of the lung volume consolidated. With the data from this individual removed, a signficant correlation of antibody titer versus % pneumonia was not present, with r=0.07 (P=0.83).

The DCPM of OMP-stimulated mediastinal lymph node cells approached a significant correlation with antibody titer on day 31, with R=0.59 (P=0.06), however the DIFN of OMP-

Fig. 5. Correlation of proliferative (A), gamma interferon (B) or antibody response (C) with % pneumonia of calves after challenge exposure to P. haemolytica 31 days after calves were vaccinated or infected with P. haemolytica. Mediastinal lymph node cells were stimulated with outer membrane protein (OMP) from P. haemolytica 35 or 36 days after calves were vaccinated or infected with P. haemolytica. Antibody titers on day 31 were determined by an indirect hemagglutination assay. The DCPM or DIFN represent the difference in CPM or ng of gamma interferon/ml of antigen-stimulated wells from background wells. Different symbols are used to identify the treatment group of the individuals. Correlation coefficients (r values) are statistically significant when P≤0.05
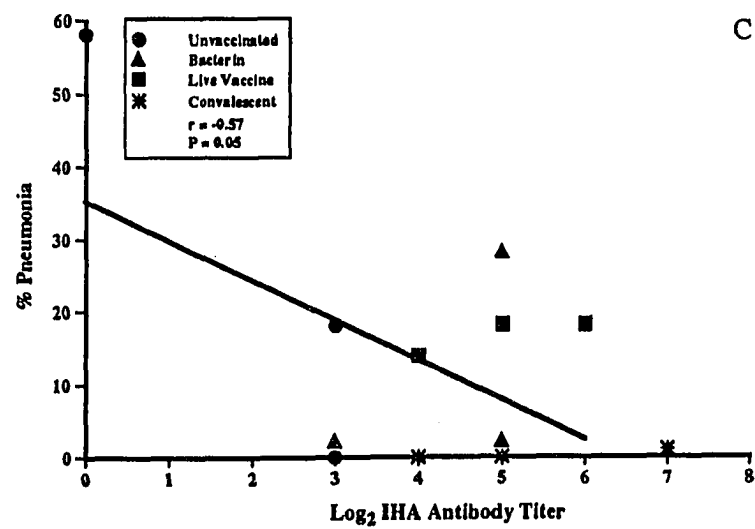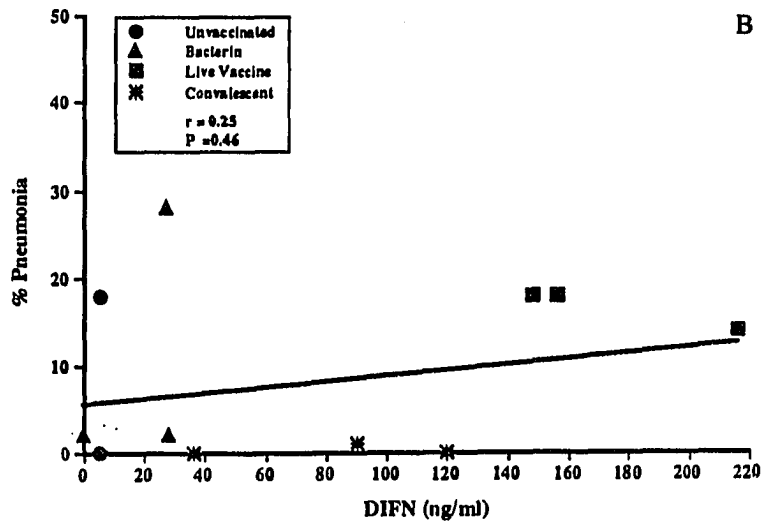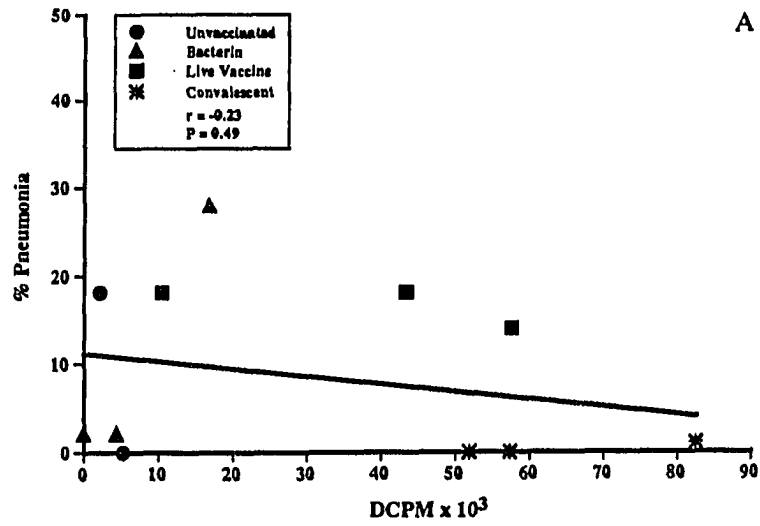
stimulated mediastinal lymph node cells did not correlate

significantly with antibody titer on day 31 (P>0.05), (Fig.

6). The DIFN of OMP-stimulated mediastinal lymph node cells

did not correlate significantly with the DCPM of OMP-

stimulated mediastinal lymph node cells (P>0.05) (Fig 6).

Fig. 6.   Correlation of proliferative or gamma interferon
          responses of calves with antibody titer (A and B)
          and correlation of proliferative responses with
          gamma interferon responses (C). Mediastinal lymph
          node cells were stimulated with outer membrane
          protein (OMP) from P. haemolytica 35 or 36 days
          after calves were vaccinated or infected with P.
          haemolytica. Serum antibody titers on day 31 were
          determined by an indirect hemagglutination assay.
          The DCPM or DIFN represent the difference in CPM or
          ng of gamma interferon/ml of antigen-stimulated
          wells from background wells. Different symbols are
          used to identify the treatment group of the
          individuals. Correlation coefficients (r values)
          are statistically significant when $P \leq 0.05$
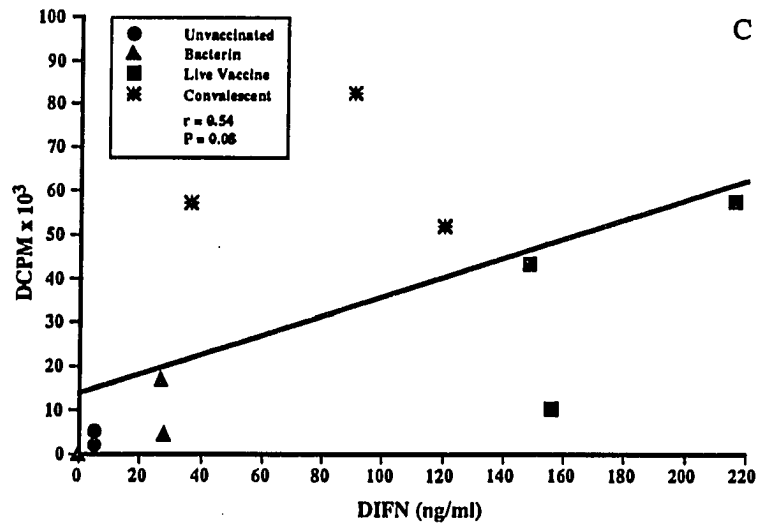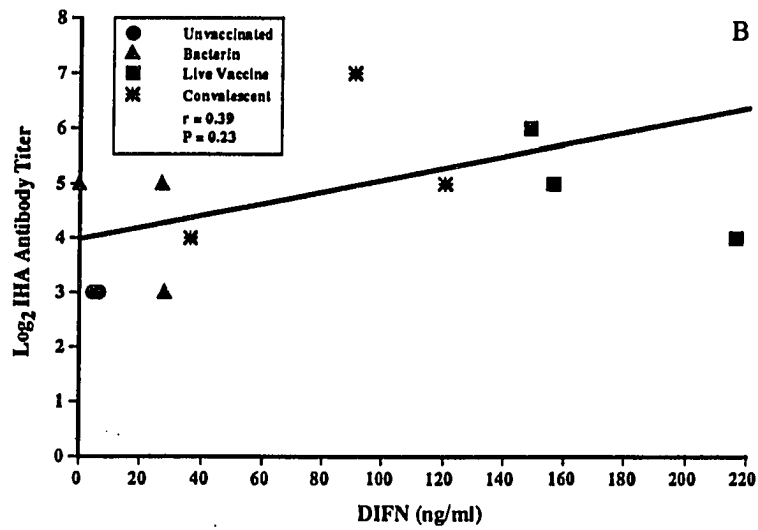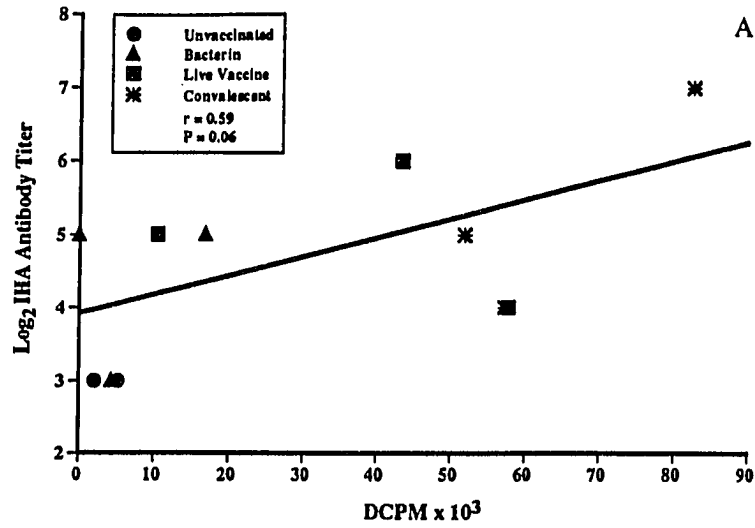
DISCUSSION

In this study, cattle vaccinated with a live P. haemolytica vaccine or infected intratracheally with P. haemolytica developed cell-mediated immune responses, as assessed by measuring proliferation and gamma interferon production by lymph node cells following incubation with certain P. haemolytica antigens. Proliferative responses were significant when OMP and SON antigens but not when CP, LPS or LAP antigens were used to stimulate lymph node lymphocyte cultures. The lack of responsiveness of PBMC cultures in the present study may reflect a lack of effective antigen presenting cells in PBMC preparations (34).

The highest proliferative responses were present in mediastinal lymph node cells of calves in the live vaccine group and the convalescent group. There was clinical evidence of pneumonia in all calves of the convalescent group and chronic pneumonia was present in two of three calves at necropsy. In cattle, the caudal mediastinal lymph node receives lymphatic flow from the lung (27), therefore, abundant P. haemolytica antigen from pneumonic parenchyma should have been delivered to the caudal mediastinal lymph node of calves in the convalescent group.

Calves in the bacterin group had higher proliferative responses in superficial cervical than in mediastinal lymph node cells, indicating that the immunologic responses were

localized near the site of administration of the bacterin. Conversely, calves in the live vaccine group had higher proliferative and gamma interferon responses in mediastinal than in superficial cervical lymph node cells, indicating that abundant antigen from P. haemolytica was delivered to mediastinal lymph nodes for sensitization of T lymphocytes. It is likely that the live vaccine replicated in the lung, or, possibly, that blood-borne antigens were removed in the lung and transported to mediastinal lymph nodes. Pasteurella haemolytica may cause pneumonia when inoculated intravenously into animals (13, 31), and it is possible that the live vaccine replicated in sites other than the site of vaccination. Administration of live P. haemolytica vaccines has been associated with P. haemolytica septicemia and meningitis in cattle (35). In the present study, calves vaccinated with the live vaccine had febrile responses after vaccination, as well as extensive tissue swelling at the site of vaccination; either may be associated with septicemia, but blood cultures were not performed on calves to determine if bacteremia occurred.

Although relatively few animals were used in the present study, proliferative and gamma interferon responses did not correlate with the severity of pneumonia after challenge. These data provide no strong evidence for the role of CMI in protection against pneumonic pasteurellosis. However, one

animal in the unvaccinated group markedly affected the correlation between antibody titer and % pneumonia. When the data from this individual was removed for statistical analysis, the correlation between antibody titer and % pneumonia became less significant than the correlations between CMI responses and % pneumonia. Confer et al. (6) demonstrated that antibody responses of calves vaccinated with live P. haemolytica correlated with protection against challenge with virulent organisms, whereas antibody responses of calves vaccinated with a killed bacterin did not correlate with protection. Numbers of calves in the present study, however, were not sufficient to allow correlations of immune responses of calves within experimental groups with protection against the challenge exposure.

As an effector cytokine of the CMI response, gamma interferon may provide assistance in elimination of P. haemolytica by enhancing phagocye uptake and killing of the organism. Gamma interferon has been shown to enhance cellular antimicrobial activities against some bacterial species, including Mycobacterium bovis (14) and Listeria monocytogenes (26). Administration of gamma-interferon to mice has been shown to reduce infection rates after challenge with Brucella abortus (28). When administered to cattle, recombinant bovine gamma interferon reduced the severity of pneumonia in dexamethasone-treated calves that were

challenged with <u>Haemophilus somnus</u> (4). In the present study, the in vitro gamma interferon response was highest in calves from the live vaccine group, although calves in this group did not have significantly less pneumonia after challenge than those in the groups with lower gamma interferon responses. Although gamma interferon has been shown to facilitate killing of intracellular pathogens, it's effect on cellular defenses against <u>P. haemolytica</u> is unknown. <u>Pasteurella haemolytica</u> survives and replicates in the extracellular environment (8); therefore, the beneficial bactericidal-enhancing effects of gamma-interferon may not be as significant for <u>P. haemolytica</u> as for facultative intracellular bacteria such as <u>Brucella</u> or <u>Listeria</u> species.

In this study, CMI responses to <u>P. haemolytica</u> were detected and quantitated in vitro, using lymph node-derived lymphocytes. Further studies are necessary to determine the role of CMI alone or in conjunction with humoral immune responses in immune protection of cattle against pneumonic pasteurellosis.

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GENERAL DISCUSSION AND SUMMARY

The first of the two studies demonstrates that the goat may serve as a suitable model for studies of bovine pneumonic pasteurellosis, with all of three goats developing pneumonia after intratracheal inoculation with P. haemolytica. Furthermore, one of three goats that was inoculated intravenously developed pneumonia, supporting the study by Thomas et al. (1989) in which cattle developed pneumonia subsequent to intravenous inoculation with P. haemolytica. Although experimental studies demonstrate that P. haemolytica can cause pneumonia when inoculated into ruminants by the intravenous route, the probability of an intravascular route of the organism to the lung in natural cases must be questioned. Pneumonia was not demonstrated in goats that were intratonsillarly-inoculated with P. haemolytica in the present study, providing no evidence of invasion of the tonsil as a route of access of the organsim to the lung. Lymphoid tissues, however, have been demonstrated to be a portal of entry of other pathogens (Ackermann et al., 1988, Momotani et al., 1988, Woode et al., 1984).

In the second study of the dissertation, there was evidence of accession of antigens of P. haemolytica to the lung after vaccination of calves with a live P. haemolytica vaccine. Proliferative and gamma interferon responses of mediastinal lymph node cells from calves vaccinated with the

live P. haemolytica vaccine indicated a likely replication of the vaccine-derived bacteria in the lung. A vascular route of the organism to the lung is likely, and ruminants have abundant pulmonary intravascular macrophages, which cause a high percentage of intravascular bacteria to be removed from the blood by the lung (Warner et al., 1987). In the present study, however, it was not determined if calves vaccinated with the live vaccine developed pneumonia.

In the second study of this dissertation, CMI responses were demonstrated from calves after vaccination with a live vaccine or after inoculation of calves with virulent P. haemolytica via the respiratory tract. The CMI responses were demonstrated either by assessment of lymph node lymphocyte proliferation or gamma interferon production subsequent to in vitro stimulation with OMP or SON antigens extracted from P. haemolytica. This is the first published study of CMI responses of cattle to P. haemolytica. Numerous studies have reported on humoral immune responses to P. haemolytica, and antibody responses to leukotoxin or antigens present on the surface of P. haemolytica have been associated with protection against pneumonic pasteurellosis (Confer et al., 1985b, Gentry et al., 1985). However, concurrent but unassessed CMI responses may have been present that may have also contributed to the protection against challenge. Although neither lymphocyte proliferative or gamma interferon

responses correlated strongly with protection against challenge with P. haemolytica in the present study, neither did antibody responses correlate strongly with protection.

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APPENDIX 1

MATERIALS AND METHODS FROM PAPER 1

Ten colostrum-deprived, crossbred goats were reared in isolation until the study was initiated when goats were 14-18 weeks of age. Pasteurella haemolytica was not isolated from nasal swabs obtained from the goats prior to inoculation with P. haemolytica. Prior to inoculation of goats with P. haemolytica, antibody levels to P. haemolytica were determined by indirect hemagglutination. Seven goats had titers ranging from 1:4 to 1:16. Goats were randomly assigned to three treatment groups of three goats per group, with one goat remaining as a sham inoculated control. Goats were housed indoors under controlled environmental conditions, with all individuals in each treatment group in a separate room. Goats were euthanatized intravenously with pentobarbital and necropsied 42-66 hours after inoculation with P. haemolytica.

Pasteurella haemolytica A1 (isolate L101) was isolated from pneumonic bovine lung and first passage colonies were inoculated into tryptose broth and frozen at -70 C. For preparation of inocula, organisms were thawed and inoculated into tryptose broth. After 6 hours incubation at 37 C, bacteria were pelleted by centrifugation at 5900 X g for 5 minutes at 4 C. Organisms were washed two times in tryptose broth, and inocula were adjusted to 80% transmittance at a

wavelength of 600 nm in tryptose broth, and were then serially diluted to inoculation concentrations. Inocula were maintained on ice until inoculated into goats, which was less than 1 hour after preparation. Concentration of organsims in inocula was confirmed by standard plate counts. Goats that were inoculated intratracheally and intravenously were given 10 ml of an inoculum containing 2.0 X 10^5 CFU of P. haemolytica, while goats that were inoculated intratonsillarly were given 0.1 ml of inoculum containing 2.3 X 10^7 CFU in each palatine tonsil. For intratracheal inoculation, an 18 gauge, 1 inch needle was inserted into the tracheal lumen approximately 3-5 cm below the larynx, and the inoculum was delivered over a period of approximately 5 seconds. Inoculum was delivered slowly into the jugular vein of intravenously-inoculated goats. Goats that were inoculated intratonsillarly were sedated with 0.25 mg xylazine intravenously, then were placed in lateral recumbency. Inoculum was placed in the external orifice of each palatine tonsil with a 1 ml plastic pipette which had the tip slightly curved and blunted to avoid traumatizing the surrounding mucosa. A laryngoscope was used to allow access to the palatine tonsils. The control goat was inoculated with sterile tryptose broth; 10 ml was given both transtracheally and intravenously, and 0.1 ml was given intratonsillarly.

Venous blood was collected from all goats in sterile sodium citrate for quantitative bacterial culture at 1 minute, 30 minutes, 2 hours, and 24 hours after inoculation with P. haemolytica. Blood was collected from the jugular vein, contralateral to the site of inoculation of intravenously-inoculated goats, and maintained on ice until cultured. Within one hour after collection, 0.1 ml of blood was spread onto each of two 5% sheep blood agar plates and incubated at 37 C for 24 hours.

Samples of lung (from gross lesions if available), liver, kidney, spleen, brain and palatine tonsil were homogenized with tryptose broth using sterile Ten Broeck tissue grinders. Ten-fold dilution of homogenized tissue were spread onto each of two sheep blood agar plates for isolation of P. haemolytica. Colonies were identified by typical morphology, the presence of typical morphology and minimal incomplete hemolysis, and rapid slide agglutination with antiserum.

Rectal temperatures were taken twice daily on all goats after inoculation, and goats were observed at least twice daily for clinical signs of disease. At necropsy, internal organs were examined for gross lesions. The percentage of consolidated lung for each goat was estimated by gross examination of the lung after removal from the carcass. Sections of lung, liver, kidney, spleen, brain and palatine

tonsil were collected in 10% buffered neutral formalin, and processed and sectioned for histopathology by standard methods. Tissues were stained with hematoxylin and eosin and examined microscopically.

APPENDIX 2

SERUM LEUKOTOXIN NEUTRALIZATION ASSAY

Serum leukotoxin neutralization titers were determined on serum samples taken from calves of the study presented in Paper 2 of this dissertation. The titers were determined using a leukotoxin neutralization assay with BL-3 cells as toxin-sensitive cells, by methods developed by R. E. Briggs, Ames, IA.

Stock P. haemolytica A1 (OSU strain) leukotoxin was thawed and titrated to determine the concentration necessary to kill 30-70% of BL-3 cells (kindly provided by S. R. Bolin, Ames, IA) that had been passaged the previous day. Additional leukotoxin was thawed and diluted to eight times the titer necessary to kill 30-70% of the BL-3 cells. In 96-well plates, toxin was added for one hour at 25 C to serum samples that were diluted in Earle's Balanced Salt Solution (EBSS, Sigma Chemical Co., St. Louis, MO). The BL-3 cells that had been washed once in EBSS were then added to wells at a concentration of 2×10^5 cells in a volume equal to the combined volume of serum and toxin present in the wells. Plates were then incubated at 37 C with constant shaking. After one hour, plates were centrifuged at 1000 X g for 10 minutes and the supernatants were discarded. 1-[4,5-dimethylthiazol-2-yl] - 3,5 diphenylformazan (MTT, Sigma Chemical Co., St. Louis, MO) was then added to the wells at

100 ug/well in 100 ul of RPMI. Cells were incubated with the MTT at 37 C, with constant shaking, for 90 minutes. Plates were then centrifuged, supernatants were discarded, and 100 ul of dimethylsulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO) was added. Plates were incubated at 25 C for 5 minutes with constant shaking, then absorbance was determined at a dual wavelength (550-650 nm). Anti-leukotoxin titers were determined at the serum dilution where 30-70% of the BL-3 cells were killed. For standards, 100% of cells killed was determined by adding sonicated BL-3 cells to wells, and 0% of cells killed was determined by including no toxin in the wells. Positive control wells were included on each plate, to which toxin was added to cells in the absence of serum. Mean antileukotoxin titers are presented by treatment group (Fig. 7), followed by correlations of antileukotoxin titers versus % pneumonia, DCPM, DIFN, and serum IHA titers to P. haemolytica (Figs. 8 and 9).

Fig. 7. Mean serum antileukotoxin antibody titers of calves

by experimental group as determined by a toxin

neutralization assay. Calves were vaccinated or

infected with P. haemolytica on day 0, and calves in

the bacterin group were re-vaccinated on day 15.

There is a significant difference ($P \leq 0.05$) between group

means within a day when a common letter is not

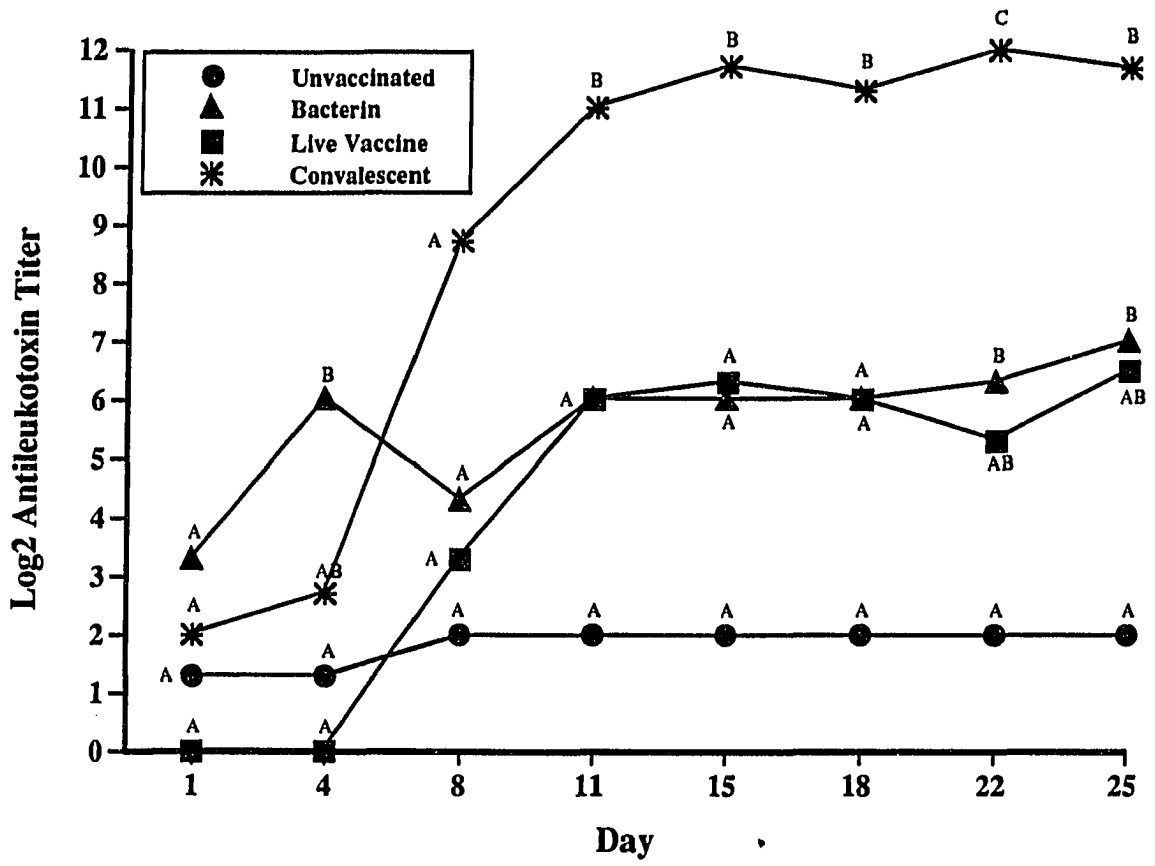present. Standard error of the means vary from 3.8

to 4.8

Fig. 8. Correlation of proliferative (A), gamma interferon
(B) or IHA antibody response (C) with antileukotoxin
antibody response of calves after challenge exposure
to P. haemolytica, 31 days after calves were
vaccinated or infected with P. haemolytica.
Mediastinal lymph node cells were stimulated with
outer membrane protein (OMP) from P. haemolytica 35
or 36 days after calves were vaccinated or infected
with P. haemolytica. Serum IHA antibody titers on
day 31 were determined by an indirect
hemagglutination assay, and serum antileukotoxin
antibody titers on day 25 were determined by a
leukotoxin neutralization assay. The DCPM or DIFN
represent the difference in CPM or ng of gamma
interferon/ml of background wells. Different symbols
are used to identify the treatment group of the
individuals. Correlation coefficients (r values)
are statistically significant when $P \leq 0.05$

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Panel A

Log$_2$ Antileukotoxin Titer

DCPM x 10^3

Unvaccinated ●
Bacteria ▶
Live Vaccine ■
Convalescent ✳

r = 0.61
P = 0.05

Panel B

Log$_2$ Antileukotoxin Titer

DIFN (ng/ml)

Unvaccinated ●
Bacteria ▶
Live Vaccine ■
Convalescent ✳

r = 0.17
P = 0.60

Panel C

Log$_2$ Antileukotoxin Titer

Log$_2$ IHA Titer

Unvaccinated ●
Bacteria ▶
Live Vaccine ■
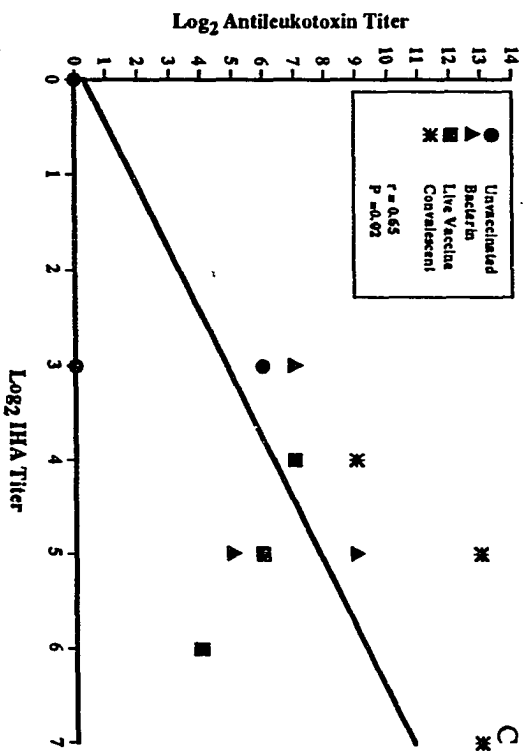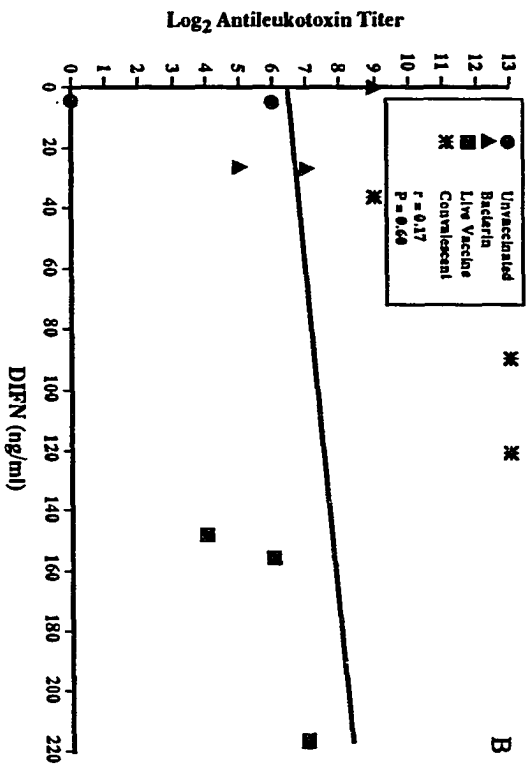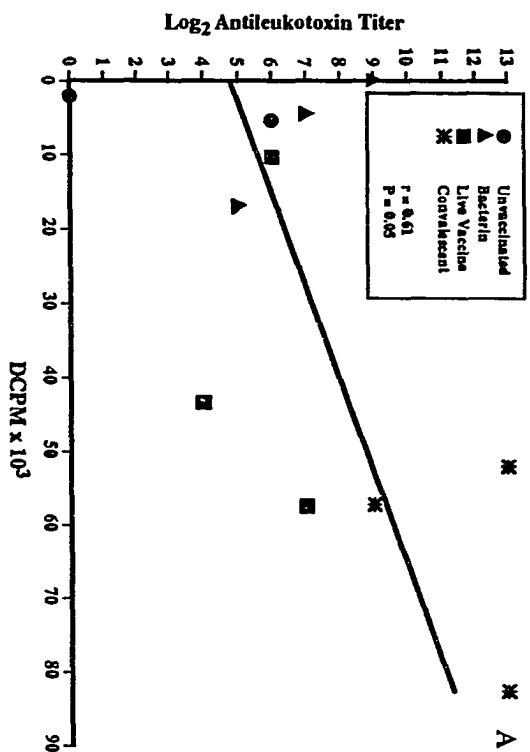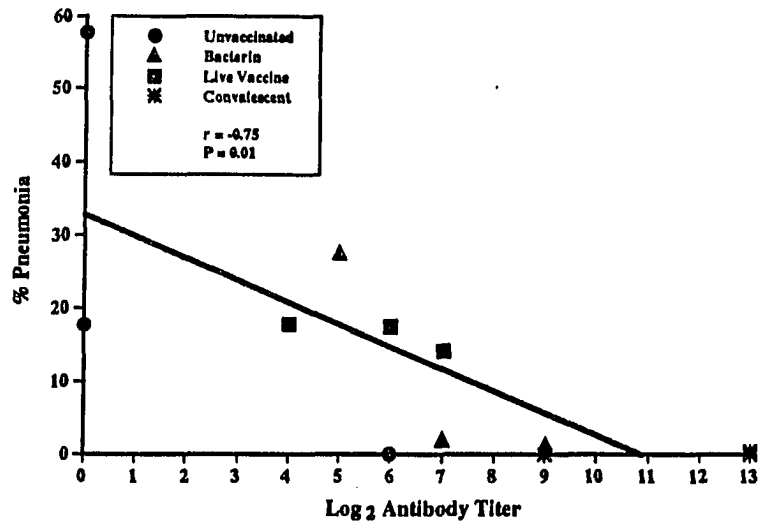Convalescent ✳

r = 0.65
P = 0.02

Fig. 9. Correlation of antileukotoxin antibody response with

% pneumonia of calves after challenge exposure to P.

haemolytica, 31 days after calves were vaccinated or infected

with P. haemolytica. Serum antileukotoxin antibody titers on

day 25 were determined by a leukotoxin neutralization assay.

Different symbols are used to identify the treatment group of

the individuals. Correlation coefficients (r values) are

statistically significant when P≤0.05

Legend:
- ● Unvaccinated
- ▲ Bacterin
- ▣ Live Vaccine
- ✳ Convalescent

r = -0.75
P = 0.01

Y-axis: % Pneumonia (0, 10, 20, 30, 40, 50, 60)
X-axis: Log₂ Antibody Titer (0–13)

APPENDIX 3

FIXATION OF BOVINE NASAL MUCUS IN SITU

Under certain conditions, P. haemolytica colonizes the
upper respiratory tract of cattle, and serves as the source
of organisms that gain access to the lung. Mechanisms of
colonization by P. haemolytica are poorly defined, although
fimbriae have been described on P. haemolytica by one group
of investigators.

The upper respiratory tract has a discontinuous mucus
blanket that overlies the epithelial cell surfaces and
entraps foreign debris and bacteria. The mucus blanket is
propelled by motile cilia, resulting in removal of entrapped
debris and bacteria.

It is not known whether P. haemolytica adheres to and
colonizes epithelial cell surfaces, or if the organism
colonizes the mucus blanket that overlies the cells. The
purpose of this group of experiments was to develop
techniques to fix nasal mucosa with in situ retention of the
mucus blanket. Once the normal mucus blanket was preserved,
morphologic studies could be performed to localize the
colonized bacteria.

In most of the experiments, anti-bovine mucus antiserum
was used to aid in retention of the mucus blanket.
Theoretically, anti-mucus antibodies should cross-link the
mucus structure to stabilize it on the epithelial cell

surface. Anti-bovine mucus antiserum was prepared in guinea pigs by the following methods. Nasal exudate was collected from the nares of a gnotobiotic calf housed in a sterile isolation apparatus. The exudate was lyophilized and resuspended in phosphate buffered saline to yeild a 2 mg/ml concentration of nasal exudate. The resuspended exudate was mixed with an equal volume of Freund's complete adjuvant and 0.1 ml was injected intradermally in 10 locations in each of 5 guinea pigs. The injection procedure was repeated 10 days later, then the guinea pigs were anesthetized and exsanguinated 7 days after the second immunization. Serum was harvested from all 5 guinea pigs and pooled. Evaluation of the antiserum by agar gel immunodiffusion revealed prominent bands of identity when the nasal exudate was used as the antigen.

Preparation of the tissues for microscopic examination was repeated by similar methods, as follows, unless otherwise noted. Briefly, the tissues were trimmed to approximately 1 X 2 micrometers, immersed in 1% osmium tetroxide for 1 hour, dehydrated in graded alcohols, and embedded in Epon. Tissues were then sectioned at 2 microns thickness and stained with toluidine blue for examination by light microscopy.

Experiment 1

A bovine head was received from a local abbatoir, and circular sections of nasal mucosa from sites on the ventral

turbinate and nasal septum were removed and immersed in the anti-mucus antiserum or saline for 2.5 hours. Sections were placed in 2.5% glutaraldehyde for 18 hours, then prepared for microscopic examination. None of three sections treated with anti-mucus antiserum or three sections treated with saline had visible mucus overlying the epithelial cells when examined.

Experiment 2

Techniques used were as those described in experiment 1, except metal serum vial covers were applied to the nasal mucosal surface of the nasal septum to serve as reservoirs for the anti-mucus antiserum, which was applied for 1.5 hours. Normal guinea pig serum was applied to additional vial covers to serve as controls. The metal covers were removed, and the mucosal sections were excised and fixed for 4 hours in 2.5% glutaraldehyde. Tissues were then prepared for microscopic examination. Four of 4 sections fixed with anti-mucus antiserum had variable amounts of mucus retained, while 0 of 2 sections fixed with normal guinea pig serum had mucus present.

Experiment 3

A calf of approximately 4 months age was euthanatized and the head was removed immediately after death. Metal serum vial covers were applied to the nasal mucosa of the nasal septum, and anti-mucus antiserum was applied to the

mucosa for 2 hours, followed by application of 2.5%
glutaraldehyde to the metal caps for an additional 3 hours.
The mucosal sections were excised and immersed in 2.5%
glutaraldehyde for 18 hours, then were prepared for
microscopy. One of 3 sections treated with anti-mucus
antiserum had a minimal amount of mucus present, while none
of the sections treated with normal guinea pig serum had
mucus present.

Experiment 4

Two calves that were inoculated with BHV-1 and P.
haemolytica were euthanatized, and their heads were removed
immediately after death. Metal serum vial covers were
applied to the nasal mucosa of the nasal septum, and
experimental procedures used in Experiment 3 were repeated.
In one calf, 4 of 9 sections treated with anti-mucus
antiserum had minimal mucus present, while none of 8 section
treated with normal guinea pig serum had mucus present.
Mucosal sections from the second calf had minimal mucus
present in 3 of 6 sections, while 1 section of 8 treated with
normal guinea pig serum had minimal mucus overlying the
epithelial cells.

Experiment 5

Two calves of approximately 7 months age were
euthanatized, and their heads were removed immediately after
death. Metal serum vial covers were applied to the nasal

mucosa of the nasal septum, and 20 ul of 10% buffered neutral formalin was applied to each serum vial cover to arrest ciliary activity. Anti-mucus antiserum was then added to the covers for 2 hours, then mucosal sections were excised and immersed in 2.5% glutaraldehyde for 18 hours. The mucosal sections were then prepared for microscopy. Mucosal sections from one calf had minimal mucus present in 5 of 9 sections, while only 1 of 10 sections treated with normal guinea pig serum had mucus present. From the second calf, one of 3 sections treated with anti-mucus antiserum had minimal mucus present, while none of 3 sections treated with normal guinea pig serum had mucus present.

Experiment 6

A calf of approximately 7 months age was euthanatized, and the head was removed immediately after death. Metal serum vial covers were applied to the nasal mucosa of the nasal septum. The covers had anti-mucus antiserum, 25% glutaraldehyde, Carnoy's fixative, 95% ethanol, or 100% acetone applied for two hours. Caps were removed, and mucosal sections were excised. Those treated with antiserum or 25% glutaraldehyde were then immersed for 4 hours in 2.5% glutaraldehyde, while all others were immersed in the same fixative that was applied to the respective ring. Sections were then prepared for microscopy. Two of 3 sections treated with anti-mucus antiserum had minimal mucus present, while 2

of 2 sections fixed with 100% acetone had a blanket of material resembling mucus overlying the epithelium. Marked cellular artifact was induced by the acetone treatment. Neither of each of two sections treated with 25% glutaraldeyhde, Carnoy's fixative, or 95% ethanol had mucus present.

Experiment 7

Two bovine heads were received from a local abbatoir. The nasal mucosa of the nasal septum from one head was sprayed with an aerosol of 37% formaldehyde. Metal serum vial covers were applied to the nasal mucosa of the nasal septum of both heads and anti-mucus antiserum was applied for two hours. Mucosal sections were then excised and immersed in 2.5% glutaraldehyde for 18 hours, then were prepared for microscopy. Two of 6 sections that were vaporized with formaldehyde and treated with anti-mucus antiserum had minimal mucus present, while 2 of 5 sections treated with anti-mucus antiserum without vaporization with formaldehyde had minimal mucus present.

Experiment 8

An adult cow was euthanatized and the head was removed immediately after death. A nebulizer delivering 2.5% glutaraldehyde was attached to the external nares to aersolize the fixative through the nasal cavity, allowing aerosolized fixative to flow out through the bisected

trachea. After aersolization for 1.5 hours, metal serum vial covers were applied to the nasal mucosa of the nasal septum, and anti-mucus antiserum, 2.5% glutaraldehyde, 50% acetone, or 100% acetone was applied to the covers for 2 hours. Mucosal sections were then preapred for microscopy. Two of 3 sections treated with anti-mucus antiserum had mucus present, one of which had a linear blanket of mucus covering approximately one-half of the section. Two of 3 sections in which glutaraldehyde was applied to the rings had a minimal amount of mucus present. None of 2 sections treated with 50% acetone had mucus present, while 2 of 2 sections treated with 100% acetone had mucus present, although artifactual changes were present in epithelial cells.

Experiment 9

An adult goat was euthanatized and the head was removed immediately after death. Sections of nasal mucosa from the nasal septum were excised and placed in isopentane that was cooled with liquid nitrogen to a point slightly above the freezing point of the isopentane. Nasal mucosal sections were then frozen in the isopentane, then transferred to acetone at -70 C and stored for 96 hours. Mucosal sections were then embedded in glycol methacrylate by standard methods, maintaining the tissue below 0 C until embedded. Tissues were sectioned at 2 micrometers thickness and stained with hematoxylin and esoin by standard methods. Three of

three sections had a blanket of mucus overlying the
epithelial cells. The mucus layer was composed of fibrillary
material, possibly because of artifactual changes induced in
the mucus by freezing.

In conclusion, methods using anti-mucus antiserum to
preserve the mucus layer for morphologic examination resulted
in increased retention of mucus overlying the epithelium, but
a consistent blanket of mucus was generally not retained.
Methods of freezing the tissue in isopentane with embedding
in glycol methacrylate were superior in retention of the
mucus layer, although artifactual changes were present in the
mucus layer.