THE PATHOGENESIS OF *HAEMOPHILUS SOMNUS* PNEUMONIA OF CATTLE

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ABSTRACT

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The bacterium Haemophilus somnus is a significant cause of bovine pneumonia which annually produces large economic losses in the USA. Little is known regarding the mechanisms by which Haemophilus somnus causes pneumonia largely because a reproducible model of the naturally occurring disease has not been described.

This research describes the gross, histologic and ultrastructural lung lesions produced by the intratracheal exposure of young calves to Haemophilus somnus. A possible mechanism of lesion development, the selective adherence of Haemophilus somnus to bronchiolar epithelium, was also examined in an in-vitro lung explant system.

By 72 hours post-exposure calves exposed to Haemophilus somnus developed a neutrophilic bronchiolitis and bronchopneumonia which morphologically resembled the naturally occurring disease. The 50% effective dose for Haemophilus somnus was 1.3×10^9 and there was significant regression ($p = 0.01$) of the number of bacteria in the

inoculum on the amount of pneumonia produced.

By as early as 1 hour post-exposure, fluid and neutrophils were observed in alveolar and bronchiolar lumens. Over the next 72 hours the bronchiolar exudates contained significantly more neutrophils ($p = 0.01$) than alveolar exudates.

Inflammatory cell migration occurred from two distinct sites; 1) the alveolar capillaries and 2) the bronchiolar submucosal vasculature. Neutrophils on the bronchiolar mucosa interdigitated with bronchiolar epithelial microvilli and cilia, and neutrophil persistence was accompanied by epithelial damage. The presence of large numbers of bacteria in focal regions of lung was associated with necrosis of inflammatory cells and pulmonary tissue.

Haemophilus somnus did not selectively adhere to nor damage bronchiolar epithelium in bovine lung explants incubated for up to 6 hours with the bacteria. Haemophilus somnus colonized alveoli in significantly higher numbers ($p = 0.01$) than bronchioles and caused alveolar epithelial detachment.

It was concluded that 1) an experimental model of Haemophilus somnus pneumonia was developed which closely resembled the naturally occurring disease, 2) bacterial dosage was positively correlated with the extent of pulmonary lesions, 3) bronchiolar lesions did not develop as a result of bacterial prediliction for these sites and 4) a role for neutrophils in bronchiolar damage initiated

by Haemophilus somnus is likely because of the demonstrated neutrophil persistence in bronchiolar areas.

DEDICATION

To my wife, Judy, for her love and support and to my children Jenny, Chris, Teresa and Carrie, who willingly shared in this experience. All our lives have been changed.

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CHAPTER ONE

A REVIEW OF THE NATURAL AND EXPERIMENTAL DISEASES PRODUCED BY *HAEMOPHILUS SOMNUS* IN CATTLE

INTRODUCTION

The bacterium Haemophilus somnus produces disease and economic losses [36] in cattle throughout the world. [2,19,29,70,71,85,91,111,120,134] Substantial losses in feedlot cattle [95,97,116,119,169] are produced by the septicemic form of H. somnus known as infectious thromboembolic meningoencephalitis (ITEME). H. somnus respiratory [1,2,29,54,85,123,144,164] and urogenital [23,25,29,81,106,107,128,149,163,165] infections existing in herds without septicemic H. somnus syndromes have been only recently recognized. Both H. somnus septicemic disease [70,76,154] and H. somnus reproductive disease [70,76,107] have been recently reviewed.

Although the incidence of H. somnus pneumonia may be higher than that of H. somnus septicemia, [2,164] respiratory infections with H. somnus have not received the same attention. As a part of the bovine respiratory disease complex, H. somnus plays a role as one of many agents contributing to this serious problem. [61,82,101] While Pasteurella hemolytica has rightfully received most of the attention given bacterial agents in the bovine respiratory disease complex, H. somnus has been ignored by some. [159,173] Haemophilus somnus pneumonias, however, exist as significant problems in herds independent of pneumonic pasteurellosis. [1,2] Vaccination with H. somnus bacterins has been widely practiced with little apparent effect [1,2] on the incidence of H. somnus pneumonia.

Limited research has been done on H. somnus pneumonia in calves. No clearly defined experimental model of the disease resembling the naturally occurring disease has been described, and virtually nothing is known of the development of the gross, histologic and ultrastructural changes in this disease.

Although a series of fairly recent experiments report the presence of possible virulence factors for H. somnus such as the selective adherence to bovine turbinate epithelium [168] or to aortic endothelium, [160] no experiments to test possible adherence to lung epithelium have been published. Since the most consistent lesion of H. somnus pneumonia is a purulent bronchiolitis, [1,2,144] it is appropriate to determine if H. somnus adheres to bronchiolar epithelium.

The goal of this research was threefold: 1) to develop a reproducible experimental model of Haemophilus somnus pneumonia in the natural host, the calf; 2) to examine the early development of the lesions of H. somnus pneumonia for clues to the pathogenesis; and 3) to determine if selective bacterial adherence to bronchiolar epithelial surfaces occurs and is of importance in lesion development.

With these research goals in mind, the following literature review focuses on the characteristics of Haemophilus somnus, the syndromes and lesions produced by the bacteria, the virulence factors of H. somnus which might have a direct or indirect effect on the bovine lung and the

attempts by others to experimentally reproduce H. somnus syndromes.

A. CHARACTERISTICS OF HAEMOPHILUS SOMNUS:

1. GROWTH AND PHYSICAL CHARACTERISTICS:

Haemophilus somnus is a non-motile [164] Gram-negative pleomorphic coccobacillus which grows best at 37-43 C under increased CO_2 or reduced oxygen environments [139] on brain heart infusion agar [48] containing 10% fresh bovine blood and 0.5% yeast extract (BHI-Y-BAP). The addition of antibiotics, [167] sodium azide, nystatin [167] and cycloheximide to this medium improves the rate of isolation of H. somnus from clinical materials. [143] By serially passaging H. somnus in increasing aerobic conditions, H. somnus can be adapted to aerobic growth. [48,77,91] Haemophilus somnus grows poorly or not at all in basal media unless the media are supplemented with either blood, serum, or yeast extract. [77] Haemophilus somnus has no requirement for X (hemin) or Y (NAD) factors. [77] Nearly all strains of H. somnus require cocarboxylase (thiamine pyrophosphate) or thiamine monophosphate but do not require thiamine. [3] According to other workers, H. somnus does not require thiamine derivatives but does require the amino acids cysteine or cystine. [100]

Haemophilus somnus grown on the more nutritious BHI-Y-BAP medium are visible at 18-24 hours as tiny translucent to opaque, round convex colonies. [164] Colony color on the red BHI-Y-BAP medium appears greyish to light yellow, but colonies picked up on white cotton tipped swabs are bright yellow. [29,51,164] This yellow color is also

apparent in saline suspended organisms and prepared fractions of H. somnus containing cell wall material. [155]

Most strains of H. somnus are non-hemolytic on blood agar, [48,164] but greening of the agar [164] beneath and immediately surrounding bacterial growth is common [29,77] after 24-48 hours growth. Hemolysins have not been described for H. somnus, and the cause of the agar change has not been identified.

The composition of the medium in or on which H. somnus is grown changes the shape of H. somnus. Fresh isolates are pleomorphic [119] on Gram-stained smears with mixtures of coccoid, short rods and even short-chained filamentous forms observed. [77] The size of H. somnus varies from 0.3-0.5 X 0.8 to 4.0 micrometers, [77] and the organism may appear bipolar. In H. somnus cultures serially passaged on artificial media, the ratio of coccoid forms to longer bacillary forms increases. [29] Growth of H. somnus in BHI enriched with IsoVitelex [100] or thiamine pyrophosphate is predominantly filamentous with chains of up to 8-15 bacilli. [3] The addition of serum allows single cell growth to occur with more coccoid forms appearing. [3]

A question remains regarding the presence or absence of a capsule surrounding H. somnus. [48,77,101,168,171] Several investigators demonstrated what they believed to be a capsule [101,171] using a modified Hiss stain or India ink. However, others have not been able to substantiate those findings using capsular stains [48,77] or electron

microscopic examinations. [168] The polysaccharide capsular stain, ruthenium red, has been used in transmission electron microscopic examinations, [168] but no one has reported using antibody or lectin to support delicate capsular material. [32] If present, the capsule of H. somnus may not survive the dehydrative processes of transmission electron microscopy specimen preparation unless supported by antibody, [32] Although polysaccharides have been extracted from the surface of H. somnus, [101] these may have been from the side chains of lipopolysaccharide or LPS (a part of the outer membrane) or from capsular material (if present), and therefore the presence of polysaccharides does not confirm the presence of a capsule.

The cell envelope of H. somnus is typical of Gram-negative bacteria composed of three layers resolvable by transmission electron microscopy [150]; 1) an outer membrane, 2) the periplasmic space and 3) the cytoplasmic or plasma membrane. A distinct peptidoglycan was not detected in these preparations but was presumed to be present. [150] Haemophilus somnus is similar morphologically to Haemophilus equigenitalium. [158]

The biochemical activity of H. somnus varies depending on the medium in which specific biochemical activities are tested. Using supplemented medium in 5-10% CO_2 atmospheres, most strains of H. somnus weakly ferment a number of carbohydrates including glucose (dextrose), maltose, mannitol, mannose, sorbitol, trehalose, xylose and levulose

and weakly produce indole. [29,48,77,91,131,139] Haemophilus somnus also reduces nitrates, weakly acidifies litmus milk and produces cytochrome oxidase. [29,48,77,116] Catalase is not produced by most strains [48,51,116,138,151] but is by some. [29]

Haemophilus somnus is sensitive in-vitro to a wide spectrum of antibiotics [29,77,91,112,131,157] and antibiotic treatment reduces the likelihood of isolating H. somnus from affected cattle. [96]

2. ANTIGENIC CHARACTERISTICS:

Haemophilus somnus antisera weakly reacts with a variety of bacterial organisms. Some of these cross-reacting organisms include Bordetella bronchiseptica, [39,139] Streptococcus agalactiae, [17,101] Actinobacillus ligniersi, [48] Pasteurella multocida, [17] Pasteurella hemolytica, [17,94] Staphylococcus aureus, [17] Moraxella bovis, [48] and Listeria monocytogenes. [101] Very sensitive methods, such as the ELISA and agglutination tests, [48] have demonstrated low levels of cross-reactivity even with relatively specific H. somnus antigen preparations. [17] However, the magnitude of homologous serologic reactions indicates a predominance of organism-specific antigen [17] for H. somnus. Closely related organisms such as Haemophilus agni and Histophilus ovis have much higher levels of cross reactivity with H. somnus than other organisms listed above. [17,39,101,151] All sera from adult sheep in one study [86] had microscopic

agglutination test titers to H. somnus, presumably because of cross-reactivity with H. agni.

Until recently, all strains of H. somnus were considered to be antigenically similar, if not identical. Using polyacrylamide gel electrophoresis (PAGE), [140] agglutination absoptive methods, [28] and ELISA methods, [28] workers have been able to identify various antigenic types of H. somnus. These serotypes of H. somnus did not correlate with the anatomical site of H. somnus isolation, [16] but geographical serogroups of H. somnus exist. [16,28,65]

B. HISTORICAL ASPECTS OF HAEMOPHILUS SOMNUS:

Although interest in the organism we now call H. somnus was generated in the 1960's when it was isolated from cattle with the septicemic disease known as infectious thromboembolic meningoencephalitis (ITEME), it is possible that the earliest reported isolation of H. somnus was from the lungs of calves with purulent bronchopneumonia. In 1918, Theobald Smith described an organism he isolated from pneumonic calf lung. [147] One "phase" of Smith's isolate was a gram negative pleomorphic coccobacillus which grew only on agar slants supplemented with pieces of lung tissues and incubated in environments with increased carbon dioxide and reduced oxygen. This isolate formed tiny (1-2 mm), slightly raised yellowish colonies on the clear supplemented agar. These characteristics are now attributed to H. somnus. Although Smith's isolate or isolates have

been regarded as an Actinobacillus sp., [70] the close similarities between Actinobacillus sp. and Haemophilus somnus and the rare isolation of Actinobacillus sp. from bovine lungs, [52,85] suggest that Smith's isolates may have been H. somnus or H. somnus mixed with Actinobacillus sp.

An astute and careful observer, Smith described the endemic pneumonia in these 4 week and older calves as "involving at least one of the smaller lobes (cephalic, ventral, azygos)--- usually all were pneumonic. When one of the affected lobes was cut across, a pearly white, thick mucoid mass slowly oozed out of the cut ends of the small bronchioles ---. The tissue was bright reddish and permeated with grayish 1-2 mm foci, closely set. Sections of the diseased lobes indicated a suppurative bronchopneumonia, with some fibrin in the most recently invaded tissues." These lesions are remarkably similar to those described in more recent reports of naturally occurring H. somnus pneumonia. [1,27,123]

From the time of Smith's report until the last decade, reports of the isolation of H. somnus from cattle came from the syndrome called infectious thromboembolic meningoencephalitis with only two exceptions. [34,44]

A report of the isolation of an organism similar to H. somnus from clinical bovine vaginitis was published in 1950 and preceeded ITEME papers by several years. [34] A Gram-negative pleomorphic coccobacillus was isolated from 80% of adult cattle with vaginitis and 25% of cattle without

vaginitis. The author successfully transmitted the infection to other cows by inoculation of scarified vulvar epithelium with vaginal scrapings from affected cows and, in two instances, transmitted it with saline suspensions of the 'Haemophilus-like' organism. [34] The naturally occurring and experimentally induced vaginitis was described as a nodular vaginitis or granular venereal disease similar to the Ureaplasma sp.-associated vaginitis described by others nearly 20 years later. [128] Since Ureaplasma sp. had not been recognized as a cause of bovine vaginitis in 1950, it is likely this report described a mixed Ureaplasma sp. and H. somnus infection. [128]

A slightly different 'Haemophilus-like' organism was isolated in 1959 from an aborted bovine fetus. [44] This report is occasionally referenced as an example of H. somnus abortion. [23] The isolate, however, varied from H. somnus in hemolysis on blood agar and in fermentation of carbohydrates and was probably Haemophilus citreus. [23]

Interest in other syndromes now known to be caused by Haemophilus somnus began to grow when, in 1956, Griner et al [53] described a problem in Colorado feedlot and pastured cattle they called "infectious embolic meningo-encephalitis". They first observed an animal with the syndrome in 1949 and studied field cases of the disease for nearly 7 years before publishing their report. During those 7 years, they necropsied 23 cattle affected with ITEME and examined the brains of 13 others. Twenty-five of the 36

cases occurred during 1955 and apparently provided the impetus to recognize the disease as a distinct syndrome. These authors hypothesized that a septicemia probably existed and suggested that "infectious embolic meningoencephalitis" was a sequel to infectious bovine rhinotracheitis and bronchopneumonia.

In 1958, a septicemic disease of sheep very similar to ITEME in cattle was reported in California.[78] A bacterium isolated from these sheep was tentatively named H. agni. No pneumonic changes were seen in the natural disease or in 23 sheep experimentally exposed to the organism. The sheep developed "similar signs and lesions as those observed in the natural disease."

Two years later, Kennedy et al[77] isolated a 'Haemophilus-like' bacterium from cattle which had died from infectious meningoencephalitis. The bacterium was isolated from a variety of tissues including brain, kidney, spleen, liver, skeletal muscle, synovial fluid, blood and lung. Seventy-five of 3000 animals in the herd died, and an estimated 400-800 developed clinical signs of ITEME between October and March. Eight animals were necropsied and multifocal 1-4 cm hemorrhagic foci were seen in the brains, along with increased pericardial and joint fluid and hemorrhage in the heart, skeletal muscle and kidneys. No lung lesions were reported.

Many of the characteristics of this organism were defined by Kennedy et al[77] and a close antigenic

relationship with H. agni demonstrated when sera from this infected herd reacted strongly with H. agni organisms used in a complement fixation test. [77] These workers also reproduced the septicemic disease in 5 calves by intravenous injection of calves with broth earlier inoculated with blood taken directly from ITEME affected cattle. No description of the lesions produced in the experimental calves, however, was given.

Kennedy et al [77] supported Griner's [53] hypothesis that ITEME was a septicemic disease and suggested that the lesions developed after capillaries were occluded by masses of bacteria, an acute vasculitis developed and the inflammatory reaction spread to the surrounding brain tissue resulting in thrombosis of larger blood vessels. The inflammatory cell response in experimentally infected calves was principally neutrophilic in early lesions. [77] An initial leukopenia at 24 hrs. post exposure was followed by a leukocytosis (neutrophilia) in surviving animals.

After demonstrating agglutinating antibodies in the sera of 81 of 83 steers from the naturally infected herd, Kennedy et al [77] concluded that "inapparent infection was the rule and clinical disease was the exception." They also stated that "although the origin and spread of the organism is unknown, there is the possibility that this bacterium may be an inhabitant of the upper respiratory tract."

These two reports [53,77] of ITEME from Colorado and

California were quickly followed by reports of similar syndromes and lesions in feedlot cattle in Kansas, [169] Illinois, [20] Iowa, [10,63] Oklahoma and Texas, [119] and Minnesota. [89] Infectious thromboembolic meningoencephalitis and other H. somnus diseases have since been reported from Canada, [91,111,134] England, [126] Italy, [19] Germany, [26,46,136] Switzerland, [29] Netherlands, [46] South Africa, [80] Japan, [110] and Australia. [85,149]

As more investigators began recognizing the syndrome, various names for both the syndrome and the organism began to appear. Weide et al [169] used Griner's name for the syndrome "infectious embolic meningo-encephalitis", Kennedy et al [77] called it "infectious meningoencephalitis", Case et al [20] used the term "embolic meningo-encephalitis", Howard and Fawcett [63] were first to call the syndrome "thromboembolic meningoencephalitis". Bicknell [10] used the same term as Howard and Fawcett but hyphenated it. Baile et al [5] believed the disease should be called "infectious thromboembolic meningomyelitis" because thrombosis of vessels was a prominent histologic feature and spinal cord involvement was frequent. They also called the disease "sleeper syndrome" [5] since feedlot operators and cowboys frequently termed the affected animals "sleepers". [169] Panciera et al [119] argued that no evidence of septic emboli was present in diseased animals but rather the pathogenesis of ITEME was one of septicemia with resultant vasculitis. They chose to use the cumbersome but more precise term a

septicemia caused by a 'Haemophilus-like' organism. [119] Subsequent reports used one of the above names for the syndrome. However, there is not universal agreement on any one name for this disease. [154]

C. CLASSIFICATION OF HAEMOPHILUS SOMNUS.

The proper taxonomic classification and the proper name for the organism called H. somnus are undecided. [3,79] The subcommittee on taxonomy of the genus Haemophilus stated that species without X or V factor requirements or without requirements for otherwise definable coenzymes should not be included in the genus Haemophilus. [9,175] Kennedy et al [77] called the organism 'Haemophilus-like' as did Panciera et al [119] and Shigidi. [138,139] Gossling [51] preferred to call it an Actinobacillus sp. because her isolates differed from A. actinoides only in the oxidase reaction. Kansas workers called it an 'Actinobacillus actinoides-like' organism [5] although the principal investigator later changed his mind. [6] Little et al [90] suggested A. actinoides was closely related, if not identical, to H. somnus and this has been supported by others. [85]

Stephens et al [151] compared 12 strains of H. somnus, H. agni, Histophilus ovis and A. seminis. They found a close cultural and antigenic relationship between these organisms with the exception of A. seminis. They suggested that H. somnus, H. agni and Histophilis ovis should all be placed in a single taxon and called the Haemophilus-

Histophilus group. [151] Organisms identical to Histophilus ovis have been isolated from cattle and organisms identified as H. somnus have been isolated from sheep. [17]

The DNA of H. somnus contains 37.3% guanine and cytosine and is therefore closely related to official members of the genus Haemophilus. [4,79] Haemophilus somnus DNA is also 46%, 58% and 43% homologous with the DNA from H. influenzae, H. parainfluenzae and A. ligneresii respectively, indicating that H. somnus is moderately related to those species. [50] Based primarily on the guanine and cytosine content of the DNA extracted from the bacteria, Baile [4] classified the bacterium as a Haemophilus sp. and gave it the name Haemophilus somnus deriving the species name from the Latin for sleep. This name was never validly published and Baile [6] later stated that "we cannot at present justify the inclusion of this microorganism in the genus Haemophilus." Between Baile's 1969 and 1973 reports the name Haemophilus somnus was used by Brown, et al [13,14] and for the lack of a better name, the use of the name Haemophilus somnus persists today.

D. SYNDROMES AND LESIONS OF HAEMOPHILUS SOMNUS INFECTIONS

Three interrelated syndromes of H. somnus infections have been described. [76,134] These are 1) the septicemic disease called ITEME, [111,154] 2) the urogenital infections of both males and females, [107] and 3) the respiratory infections manifested primarily by pneumonia. [1,2]

The septicemic form of H. somnus infection primarily affects cattle from 6-24 months old [5,14,89,95,110,119,169] although it has occasionally been reported in younger calves. [46,134] It rarely affects adults. [110,146] The syndrome is most common in winter months [2,77,134] in feedlot operations [5,12,89,95,97,119,133,169] and has also been reported in pastured calves. [53,146] Infectious thromboembolic meningoencephalitis is relatively uncommon in dairy herds. [89]

Gross lesions of H. somnus septicemia include multifocal hemorrhage and necrosis in a variety of tissues including the brain, spinal cord, synovia, retina, skeletal muscles, cardiac muscles, intestine, urinary bladder, kidney, liver, esophageal mucosa and lungs. [5,13,14,40,42,116,119,154,158] Brain lesions vary from diffuse fibrinopurulent meningitis [5,46,71,119,154,169] to more common multifocal hemorrhagic necrosis. [5,10,14,20,60 116,119] Concurrent fungal infection may accompany brain lesions. [137] In addition, serofibrinous arthritis or synovitis involving many peripheral joints, [5,14,60,119] diffuse fibrinous pleuritis [5,13,119] and pericarditis, [5,62,119] and bilateral laryngeal necrosis [14,40,119] are common lesions associated with ITEME. Rhinitis and bronchopneumonia [14, 169] have also been included as lesions of septicemic H. somnus syndrome, but clear documentation that they are lesions of H. somnus septicemia is lacking.

The characteristic microscopic lesions of H. somnus

septicemia are necrosis, fibrinous thrombosis and neutrophilic infiltration of the media of arterioles, arteries and veins in most body organs. [5,30,39,77,110 119,146] This lesion may be induced by disseminated intravascular coagulation [110] mediated through endothelial damage and activation of Hageman factor (Factor XII). [90,160] How H. somnus becomes septicemic has not been determined.

Although many have suggested that the septicemic disease follows an initial respiratory infection, [12,14 89,134] others have challenged this idea. [1,2,62,164] Only one report has been published of a single calf which developed lesions resembling ITEME following exposure to H. somnus via respiratory routes. [30] Cattle dying of H. somnus septicemia rarely have an active H. somnus pneumonia. [1,2,110,126,164]

Haemophilus somnus can be isolated from normal [69,106 167] and inflamed [102,149] urogenital organs of both males and females and has been incriminated as a cause of abortions, [23,81,134,163,164] weak calf syndrome, [18,148,165] early embryonic death, [81] post parturient metritis, [29,107] vaginitis, [120,164] and mastitis [59] in the female. In the male, epididymitis [102] and orchitis [102] have been attributed to H. somnus. Haemophilus somnus is commonly isolated from the vaginal tract [106,128,143,167] and cervix [149] of both normal and "problem" cows, [29,118,120,128,149, 163] the prepuce of bulls and steers, [68,69,120,164,167] and the semen of bulls. [72,83]

Urogenital shedding in uterine and vaginal discharges, urine [69,153], semen [68,72] and preputial fluids [69] may provide infective material for both respiratory and other urogenital infections. Haemophilus somnus can survive for up to 5 days at 3 C in vaginal mucus but less than 24 hours in urine. [37] Spread of H. somnus from the reproductive tract to other organs has also been suggested, [107] but that has not been documented experimentally. Haemophilus somnus may spread to the urogenital tract from an H. somnus septicemia or pneumonia. [30,114,153]

E. HAEMOPHILUS SOMNUS RESPIRATORY INFECTIONS .

Respiratory infections occuring in the absence of septicemic lesions or reproductive problems are a frequent clinical manisfestation of H. somnus infection. [1,2] As high as 29% of bovine pneumonias in calves under 1 year old may be in part due to H. somnus. [54] Clinical signs include tachypnea, dyspnea, coughing and elevated temperatures. [2] In many herds, death due to pneumonia may be the first sign noted. [2] The respiratory form of H. somnus infection occurs in calves as young as a few weeks old [2,123,164] to adult animals. [164] Haemophilus somnus pneumonia is most common in dairy calves under 200 kg [2,52,134 164] and in beef calves under 300 kg. [2,134]

The major lesions of the respiratory syndrome are purulent to necrotizing bronchiolitis [1,2,144] and bronchopneumonia [1,2,29,54,123,126,135,144] of the ventral portions

of mainly the cranial lobes. Tiny (1-2 mm) grey to white foci [2,123] are scattered throughout collapsed atelectatic lung. [1] Histologic identification of these foci confirms that they are purulent [1,2,54,123] and necrotic [1,85] bronchioles. In more chronically affected calves, peribronchiolar fibrosis [2,54,144] and bronchiolitis obliterans [1,2] are common. Diffuse interstitial pneumonia in caudal and dorsal lobules is frequent and is probably the result of concurrent viral infections. [1,2] In chronicallly affected calves, focal to diffuse hemorrhagic fibrinous to fibrous pleural tags occur [83,164] along with proliferative polypoid tracheitis. [14,41] Bilateral laryngeal necrosis [12,40] is occasionally reported but is more likely to occur with H. somnus septicemia. [12] Tracheobronchial and mediastinal lymph nodes are edematous [2] and slightly enlarged but are not usually hemorrhagic. [2]

Upper respiratory lesions attributed directly to H. somnus are not well documented. [14] Haemophilus somnus can be isolated from tracheas and nasal passages of sick [40] and healthy [31] cattle. Nasal shedding of H. somnus is variable and is usually found in less than 10% of the animals in most [33,93,116,124,132] but not all [14,57,167] infected herds. Concurrent viral infection raises the level of H. somnus nasal shedding. [33] Because H. somnus survives for up to 70 days in nasal mucus at 3 C and 23.5 C, [37] nasal shedding may be an important method of transmission.

Haemophilus somnus has also been isolated from the

conjuctiva [84] of a calf with conjunctivitis and corneal opacity and from the middle ear of feedlot cattle with otitis [113].

Depending on the serologic test used, variable numbers of cattle with titers to H. somnus are detected. [39,101,129,130] Extremely sensitive tests, such as ELISA [22,94] or agglutination tests, [24,62,101,112,129] demonstrate H. somnus titers in a high percentage of normal cattle as well as in cattle from herds with H. somnus problems. Less sensitive tests, such as the complement fixation test (CFT), detect much lower percentages of positive animals. [14,15,39,92,127] Titers, as measured by all these tests, rise significantly following H. somnus infection. [15,39,92,127,170] Protection against H. somnus challenge is not well correlated with serologic titers to H. somnus. [155] Two precipitin lines on agar gel immunoprecipitin tests [57,124,171] correlated with protection from ITEME but ELISA, CFT and agglutination titers [155] did not.

The interrelationship among the distinct syndromes of H. somnus infections in cattle are as yet unclear although both nasal and urogenital shedding of bacteria occur and may serve as a sources of infection. In addition, internal spread of H. somnus via the blood stream may occur under certain undefined circumstances. Haemophilus somnus isolates from one syndrome may produce lesions in other systems although they vary in this ability. [68] Likewise,

ITEME isolates may inhabit urogenital sites without a loss of pathogenicity for the central nervous system. [68] It is, therefore, conceivable that H. somnus isolates from any of the three syndromes may produce lesions of the other syndromes.

F. HAEMOPHILUS SOMNUS VIRULENCE FACTORS POSSIBLY ACTIVE IN BOVINE LUNG

Several virulence factors may aid H. somnus in producing disease. These include; 1) exotoxins, 2) endotoxin, 3) adherence to epithelial and endothelial surfaces, 4) chemotaxis of neutrophils, 5) interference with phagocytosis and intracellular killing, and 6) resistance to serum killing.

Based on their observations of endothelial contraction and desquamation in carotid artery organ cultures inoculated with H. somnus, Thompsom and Little [160] suggested the possibility of H. somnus exotoxin activity. Humphrey [67] induced similar changes in cultured endothelial cells within 5 hours after inoculation with live H. somnus. Because killed H. somnus, sonicated H. somnus and culture filtrates did not produce endothelial cytotoxicity, they suggested that this effect was not due to an exotoxin. [67] Haemophilus somnus cells are toxic to alveolar macrophages at a ratio of 10 H. somnus cells to 1 alveolar macrophage only after the bacteria are phagocytized, [88] again suggesting that H. somnus cytotoxicity is not related to

exotoxins.

Endotoxin has been extracted from H. somnus by a variety of procedures, [21,22] and its antigenicity and effects in classical endotoxin assays such as lethality in mice and chicken embryos, Limulus amebocyte lysate assay and pyrogenicity in rabbits has been documented. [21,22] The effect of H. somnus endotoxin on bovine lung, however, has not been identified. Endotoxin in the blood of cattle infected with H. somnus has been demonstrated. [56]

Although intravenous administration of endotoxin from Gram-negative organisms to calves resulted in massive neutrophil sequestration in alveolar capillaries and increased fluid leakage into alveoli, [117] no report of damage induced by the intratracheal administration of endotoxin in calves has been published. In sheep, endobronchial deposition of Pasteurella hemolytica endotoxin induced diffuse fibrinopurulent inflammation, edema, hemorrhage and necrosis. [11] Endotoxin was also cytotoxic to cultured bovine monocytes in a time and dose related manner. [7] Endotoxin may also injure lung indirectly by activating complement via both classical and alternate pathways [172] although complement activity is generally absent from bronchoalveolar lavage fluids. [43] Complement components C4, C2, C3 and C5 are in low or undetectable amounts in bovine serum. [8] Whether H. somnus endotoxin is cytotoxic to monocytes or can activate complement has not been reported.

Chemotaxis and activation of neutrophils by H. somnus

factors has not been directly demonstrated but is a possibility. Bovine neutrophils do not respond to formylated oligopeptides produced by most bacteria but do respond to other substances produced by Gram-negative bacteria.[45] Proteases and oxygen radicals released from actively phagocytizing neutrophils contribute to epithelial and endothelial damage in the lung.[99,161,162,172] The early lesions of P. hemolytica pneumonia in cattle are ameliorated by reducing the number of neutrophils in calves with hydoxyurea.[145]

Interference with neutrophil ingestion of S. aureus is produced by a large molecule (MW > 300,000) isolated from H. somnus cell wall material.[22,64,66] Also, a small molecule (MW < 10,000) has been identified on the surface of H. somnus which reduced in-vitro iodination of proteins via neutrophil myeloperoxidase-H_2O_2-halide system.[22,64,66] Whether these factors reduce the neutrophils' ability to phagocytize and kill H. somnus in the lungs in-vivo has not been determined.

Haemophilus somnus is readily phagocytized by bovine neutrophils in in-vitro systems but no evidence of intracellular killing of H. somnus by these neutrophils was observed.[35,58] Phagocytosis occurred in the presence of immune bovine serum both in the presence and absence of complement.[35,58] Likewise, bovine peripheral blood monocytes and alveolar macrophages readily ingested H. somnus in the presence of immune serum but were unable

to effectively kill the organism in-vitro. [87]

Immune bovine serum with complement activity exerts a killing effect on H. somnus. [141] In another study normal and heat inactivated bovine serum had little killing effect on clinical isolates from ITEME [121,142] and pneumonia but did kill 25% of the vaginal isolates. [121] The classical complement pathway, probably mediated by antibody, [121,141] and to a lesser extent the alternate complement pathway contributed to H. somnus killing. [121] Rabbit lyzozyme did not enhance H. somnus killing, but the addition of iron improved the survivability of serum sensitive strains of H. somnus. Cationized ferritin binds to H. somnus surface components and to amorphous material which streams from the bacterial surface. [166]

Adherence to epithelial cells may prevent mucociliary or phagocytic removal of Haemophilus somnus or other bacteria from the respiratory tract. [47] Virulent strains of H. somnus adhere to bovine turbinate epithelium better than non-virulent strains of H. somnus in-vitro. [168] Likewise, H. somnus adheres to carotid artery endothelium in-vitro better than Eschericia coli or Salmonella typhimurium. Haemophilus somnus adherence is not mediated by pili, fimbriae or other apparent surface structures. [160, 168] Whether selective adherence to pulmonary epithelium occurs with subsequent localization and colonization has not been determined.

Other bacteria, such as the Gram-positive cocci, Micrococcus [93] Staphylococcus and Corynebacterium sp., found in the nasal, preputial or vaginal flora may enhance H. somnus growth in-vitro. [26,93] Bacillus sp. and perhaps other microorganisms [47] compete with H. somnus on upper airway surfaces and may prevent adherence and colonization of H. somnus to mucosal surfaces.

G. EXPERIMENTAL ATTEMPTS TO REPRODUCE HAEMOPHILUS SOMNUS SYNDROMES

1. EXPERIMENTAL HAEMOPHILUS SOMNUS DISEASE IN NON-BOVINE SPECIES

Exposure of non-bovine species to H. somnus by a variety of routes at varying dosages with an assortment of pretreatments has been reported. In most experiments, little or no evidence of disease [38,51,116,119, 138,174] occurred. In others, meningitis, [19] septicemia, [77,138] peritonitis, [77,138] endotoxemia, [104] and orchitis [38] resulted.

The most significant lesions produced in non-bovine species included purulent meningitis in mice, [77,119] bacteremia in rabbits, [77,138] bronchopneumonia in dexamethasone-pretreated rabbits (Andrews, JJ; unpublished data), orchitis in hamsters, [38] endotoxemia and subcutaneous abscesses in sheep, [104] meningitis in 12 day chicken embryos, [115] and peritonitis in guinea pigs. [77] . In most experiments, dosages of 10^7 to 10^9 organisms were

necessary to produce lesions. None of these is a suitable model for studying the pneumonic disease due to H. somnus as seen in calves.

2. EXPERIMENTAL HAEMOPHILUS SOMNUS SEPTICEMIA AND REPRODUCTIVE INFECTIONS IN CATTLE.

Although inflammatory changes in various organs, including the meninges, have been produced experimentally by exposing cattle by IV routes, [73,77,114,116,119,171] the infarctive vasculitis typical of ITEME has not been produced so readily or consistently. [30,39,40,174] Because detailed descriptions of the lesions were lacking, many results of previous experiments are difficult to interpret and to determine whether the lesions produced were those of ITEME.

In the best documented reproduction of the typical lesion of H. somnus septicemia, including the brain lesions of ITEME, Stephens et al [152,153] induced changes in 16 of 23 (70%) cattle injected IV with cerebrospinal fluid taken from a 2-week-old donor calf given intracerebrally 5 ml of phosphate buffered saline (with 0.1% gelatin added) containing approximately 10^5 CFUs/ml of H. somnus (strain 43826) which had been grown for 24 hours on brain heart infusion base agar supplemented with 7% bovine blood and 0.5% yeast extract. Donor calves were killed in-extremis at 22-24 hours post-exposure and cerebrospinal fluid harvested. The cerebrospinal fluid contained 1-5 X 10^8 CFUs

H. somnus/ml. Within 1 hour of preparation, 1 ml of the cerebrospinal fluid was injected into the jugular vein of 4-18 month old calves.

Gross and microscopic lesions typical of H. somnus septicemia/ITEME were described. Grossly "red-brown foci of hemorrhagic necrosis (0.1 to 3 cm in diameter) were seen in 11 of the 16 cattle that died." Microscopically all 16 cattle had vasculitis, thrombosis, microabscessation and focal necrosis in the brain. Two cattle had extensive severe meningitis with only limited encephalitis while the other 14 had lesions scattered throughout the brain. [153]

Microscopic lesions of vasculitis, thrombosis and microabscessation were also seen in numerous organs including spinal cord (13/16), myocardium (11/16), skeletal muscles (11/16), kidney (11/16), retina (9/16), gastrointestinal mucosa (6/16) and urinary bladder (5/16). The lung lesions (if there were any) were not described, and lesions of laryngitis or laryngeal necrosis were not observed. [153]

Haemophilus somnus was recovered from multiple tissues with brain and cerebrospinal fluid consistently yielding the highest rate of recovery and the highest titer of bacteria ($10^{5.8}$ CFU's/gm of brain tissue). Haemophilus somnus was isolated from the kidney and urine in 11 of 13 calves. No H. somnus isolates were made from the nares or conjunctiva of the calves with clinical disease. Haemophilus somnus was isolated from lungs of 6 of 11 calves. [153]

Haemophilus somnus isolates from the brain, prepuce and seminal vesicles varied in their ability to produce ITEME in this IV cerebrospinal fluid model. [68] Haemophilus somnus isolate 43826 (originally isolated from brain) established itself in the prepuce and maintained its virulence for the brain. [68]

Thus a reproducible model of H. somnus septicemia/ITEME has been developed and described. [152,153] It is of interest to note that inclusion of foreign or host material (i.e. brain tissue by Young, et al [174] egg yolk by Dillman [40] and Diercks, [39] or cerebrospinal fluid by Stephens [152, 153]) was necessary for production of consistent and typical H. somnus septicemia/ITEME by IV injection. With a few exceptions, at least 1 X 10^8 H. somnus organisms were necessary to produce ITEME lesions by IV exposure. Whether growth of the organism in living media is necessary for selection or maintainence of virulence factors or whether certain products derived from living tissue need to be included in the inoculum has not been determined. No consistent lung lesions were described, and thus this model is not appropriate for studying the respiratory aspects of H. somnus disease.

Haemophilus somnus instilled into the vagina produces inflammatory changes [81,105] but does not produce fetal loss unless the organism gains entrance into the uterus [75,108, 165] or enters the fetus. [170] Although no pneumonias were reported, intratracheal exposure of pregnant cows to

H. somnus resulted in abortions.[170] Exposure of cattle to H. somnus via the reproductive system, likewise, does not appear to be an appropriate method to study H. somnus pneumonia.

3. EXPERIMENTAL HAEMOPHILUS SOMNUS PNEUMONIA.

Haemophilus somnus pneumonia has been produced in cattle by intranasal or aerosol routes of exposure in only 1 of 52 calves.[14,30,77,92,114,116,127] Intratracheal instillation resulted in pneumonia in 11 of 11 calves in three different experiments.[30,39,124] Haemophilus somnus was isolated from the lungs in pure cultures in only 3 of these eleven calves. Since the experimental group size receiving any one H. somnus isolate at a given dose intratracheally was so small, little can be concluded regarding the appropriate dosage or isolate of H. somnus needed to produce pneumonia in cattle.

Kennedy et al[77] did not produce clinical disease in 2 calves intranasally and intraocularly exposed to H. somnus. Olander et al[116] instilled 15 ml of H. somnus inoculum intranasally into 2 calves and produced transient fever in 1 without other signs of disease in either.

Brown et al[15] followed IV exposure of 6 calves to H. somnus with intranasal exposure to 5.6×10^6 H. somnus in diluted egg yolk suspension. All calves survived the IV challenge for at least one month after the initial exposure. No details of illness, lesions or other evidence of

disease were reported. Dillman [40] produced laryngeal necrosis in 4 of 6 calves, along with ITEME lesions, by IV injection of an unspecified number of H. somnus grown in and suspended in yolk sac material.

Rosiles et al [127] sprayed 2.5 ml of a suspension containing 5 X 10^5 H. somnus cells/ml and 1 X 10^6 plaque-forming units of IBR virus into each nostril of 32 cross-bred calves weighing an average of 192 kg. Complement-fixing antibodies to H. somnus were produced in H. somnus exposed cattle, but no clinical disease attributed to H. somnus was seen, and no H. somnus was isolated from the blood of these cattle. The cattle were not examined by post-mortem techniques.

MacDonald and Little [92] also exposed calves intra-nasally with H. somnus. They used five 6-8 month old bull calves and administered 10 mls of a suspension containing 1.0 X 10^7 H. somnus of encephalitis origin, weekly passaged on chocolate agar for 2 months, grown in eggs once and then plated on blood agar just prior to exposure. Three of the 5 calves developed transient fevers but no other clinical signs, and only 3 had slight post-exposure CFT antibody rises to H. somnus. No necropsy findings were given.

Nayar et al [114] gave 2 calves (weighing 250-275 kg) 1.8 X 10^{10} H. somnus suspended in 100 ml of saline aerosolized for 6 minutes and gave 1 smaller calf (weighing 200 kg) 9 X 10^9 H. somnus in 50 ml saline aerosolized for 3 minutes. No pneumonic changes were produced in any of the

calves. Aerosol exposure did produce signs of depression, ataxia, head shaking, bloat and constipation in the 2 calves exposed for 6 minutes, and H. somnus was isolated from the blood 24 hours post-exposure. No abnormal changes in white blood cell counts or distributions were found in either calf. None of the calves exposed by aerosol routes were necropsied, and no respiratory system signs were observed.

Diercks et al [39] infected three 4-6 month old beef calves with an unspecified amount of an egg yolk material containing 10^6 to 10^7 H. somnus (strain 1229)/ml intratracheally. No control calves receiving similar amounts of egg yolk material IT without H. somnus were reported. Severe consolidation of the lungs, particularly the dependant portions, occurred in 2 of these 3 calves. Two calves also developed cellulitis in the neck at the site of the intratracheal injection. All three calves died within 3 days of exposure. None had CFT antibodies to H. somnus prior to exposure. These calves may have also developed septicemic lesions, but this was not clear from the report. Likewise, 2 other calves receiving other strains of H. somnus were exposed IT and developed "less severe signs and lesions".

In one of the few well-documented attempts to produce pneumonia in calves with Haemophilus somnus, Corboz and Pohlenz [30] gave 2-week-old calves 16-18 hour growth of H. somnus in egg yolk material by intranasal, intratracheal

and IV routes. Varying dosages of 6 different strains of Swiss isolates from pneumonic calf lungs (strains 326, 449, 562, 643, 724 and 749) were given 10 calves by 1 of the three exposure routes. No calves received egg yolk without H. somnus intratracheally or intranasally. Five calves received 1 of 2 USA strains (8025-Iowa and M677-Colorado) by one of the above routes. Four calves received 4 different strains of H. somnus (USA strain M677, Swiss strains 449, 643 and 724) intratracheally at varying dosages (7.45×10^8, 1.1×10^9, 3.35×10^8 and 1.9×10^8, respectively). Haemophilus somnus was isolated from the lungs of all four calves at necropsy 3-7 days later. Haemophilus somnus only (with no other bacteria) was isolated from the lungs of 2 calves while H. somnus plus P. multocida, C. pyogenes and Mycoplasma sp. were isolated from a third calf and H. somnus plus C. pyogenes from the fourth. Haemophilus somnus became bacteremic in the calf given the USA strain IT, and H. somnus was isolated from this calf's blood 36-48 hours post-exposure and from brain, blood and other organs at necropsy. Haemophilus somnus was also isolated from the nares of 2 calves given Swiss strains IT from days 6-7 post-exposure.

Subacute purulent bronchopneumonia with extensive abscess formation was described in the calves exposed by IT routes. These changes were seen in multiple lung lobes and were extensive. The pale red to brown-red, lobularly limited pneumonic consolidation was interspersed with variable

large, partly confluent microabscesses and abscesses. The bronchi were filled with mucoid yellow-brown exudate. In regions of abscess formation, the formation of thrombosis, extended leukocyte stasis and intramural and perivascular fibrin exudation predominated.

Intranasal exposure of 3 calves to 4.2×10^8 organisms (2 calves with USA strain M677) and 6.0×10^8 organisms (1 calf with Swiss strain 643) produced relatively mild pneumonic changes in only 2 calves, and H. somnus was reisolated from the lungs of only one (the Swiss strain). Nasal shedding of H. somnus was detected from these calves only on exposure day and not at any time thereafter. No evidence of bacteremia developed.

The authors reported that all calves exposed to H. somnus (by IV, IT and IN routes) developed "toxic shock"-like symptoms including increased body temperature, dyspnea, somnolence, and recumbency within 3 hours post-exposure. These symptoms disappeared by 8-10 hours later. A marked neutropenia occurred within one-half hour to 3 hours post-exposure and by 8 to 12 hours post-exposure these values were normal. Neutrophilia resulting in leukocytosis occurred 18-25 hours post-exposure. The number of platelets, levels of plasma fibrin, blood coagulation times, serum glutamic pyruvate transaminase and alkaline phosphatase did not vary remarkably over the course of the disease. In at least 2 calves, IT exposure to H. somnus in the range of 3.35 to 7.45×10^8 organisms suspended in 5

mls of egg yolk material produced suppurative broncho-pneumonia in young calves without the assistance of other bacterial agents.

Pritchard [124] intratracheally infected two 48-55 day old dairy calves with 5 X 10^9 and 1.4 X 10^{10} H. somnus (second passage from pneumonic lung-strain DB127/76) suspended in 10 mls of 'E' medium. One calf developed severe cellulitis at the site of IT injection and acute suppurative bronchopneumonia characterized by suppurative alveolitis, bronchitis, bronchiolitis, alveolar congestion and edema, local hemorrhage and severe interlobular fibrinous edema. Both P. hemolytica and H. somnus were isolated from this lung and H. somnus was isolated from heart blood. The other IT exposed calf developed multiple abscesses at the "roots of the bronchi", and H. somnus was isolated in pure cultures from these abscesses and the trachea. Both calves developed neutropenia followed by leukocytosis. Haemophilus somnus was also isolated [124] from the nasal cavities of both intratracheally exposed calves within 5 hours post-exposure and "became part of the resident flora."

Suppurative pneumonia characterized by necrotizing bronchiolitis, interlobular and alveolar accumulation of fibrin, neutrophils and macrophages and variable coagulative necrosis, arteriolar thrombosis and necrotizing vasculitis was produced in an unspecified number of dairy calves challenged intrabronchially with 10^7 to 10^9 of a

respiratory isolate of H. somnus.[49] Nine adult cows challenged IT with 2 X 10^{10} H. somnus (an abortion isolate) did not develop pneumonia.[170] Five calves exposed IT with 1 X 10^{9} CFU's H. somnus originally isolated from a pneumonic lung had significantly more pneumonia at 6 days post-exposure than calves similarly exposed to brain (5 calves) or preputial (5 calves) isolates of H. somnus.[55] Eight calves exposed intrabronchially with an unspecified dose of H. somnus developed acute to chronic necrotizing, suppurative, lobular bronchopneumonia and pleuritis.[122] Pneumonia was more severe in 4 calves given IBR virus 4 days prior to exposure to H. somnus and in 13 calves given bovine respiratory syncytial virus (BRSV) 8 days prior to H. somnus.[122] Further details of the lesions or exposure methods used to produce the pneumonia in these 4 experiments[49,55,122,170] have not been published.

In summary, exposure of calves to Haemophilus somnus by respiratory routes in an attempt to reproduce the pneumonic syndrome has been less than successful. Intranasal or aerosol instillation of H. somnus into a total of 52 calves (2 by Kennedy,[77] 2 by Olander,[116] 10 by Brown,[14] 32 by Rosiles,[127] 5 by MacDonald,[92] 3 by Corboz,[30] 3 by Nayar[114]) produced pneumonia in only 1 from which H. somnus was subsequently was isolated. Haemophilus somnus dosages for IN exposure varied from 5 X 10^{5} to 6.0 X 10^{8}, and the aerosol dose varied from 9 X 10^{9} to 1.8 X 10^{10}. The only pneumonia produced was with H. somnus grown in and suspended in

egg yolk material at the highest dose (6.0×10^8). [30]

Intratracheal exposure of calves to H. somnus was much more successful with pneumonia produced in 11/11 calves (5 by Diercks, [39] 4 by Corboz, [30] 2 by Pritchard [124]). Dosages of H. somnus grown and suspended in egg yolk and given intratracheally ranged from 10^6 to 10^7 [39] to 1.9×10^8 to 1.1×10^9. [30] Dosages of H. somnus in 'E' medium used to produce pneumonia ranged from 5×10^9 to 1.4×10^{10}. [124] Haemophilus somnus was isolated from the pneumonic lungs of only 6 of these 11 calves. The H. somnus isolate was mixed with P. multocida, P. hemolytica, and/or C. pyogenes in 3 of these 6.

This leaves only 3 published examples of uncomplicated H. somnus pneumonia that have been experimentally reproduced, and in all three cases, foreign material (egg yolk or 'E' medium) was instilled into the lungs along with H. somnus organisms. An additional 23 calves, [55,122] plus an undisclosed number of calves, [49] have been exposed intratracheally or intrabronchially to H. somnus and with the production of pneumonia. These last experiments have been reported only in meeting abstracts, and details of the lesions, microbiologic findings and exposure procedures have not appeared in complete publications.

EXPERIMENTAL RATIONALE

Haemophilus somnus infections in calves are generally manifested by pneumonia [1,2]. Haemophilus somnus pneumonia differs from the more common P. hemolytica pneumonia in its characteristic purulent bronchiolitis and its less intense alveolar reactions. Virtually nothing is known of the specific mechanisms by which H. somnus induces the bronchiolitis and bronchopneumonia although in-vitro experimentation has suggested several pathogenic mechanisms. This lack of in-vivo information is, in part, because a reproducible experimental model of the disease in calves has not been described. A series of three experiments was designed to: 1) determine whether and at what dose intratracheal exposure of calves with saline suspended H. somnus organisms would result in the development of typical H. somnus pneumonia, 2) to determine what early histologic and ultrastructural events occur following H. somnus exposure, and 3) to determine if selective localization of H. somnus on bronchiolar epithelium plays a role in the development of the characteristic bronchiolitis of H. somnus pneumonia.

In the first experiment, two hypotheses were tested:

1. Haemophilus somnus suspended in saline and injected intratracheally in calves produces a pneumonia which closely resembles the naturally occurring disease by 72 hours.

2. The amount of pneumonia produced experimentally by

intratracheally exposing calves to H. somnus is directly related to the number of viable bacteria in the inoculum.

In this first experiment, 15 one to four-week-old bull dairy calves were inoculated intratracheally with varying numbers of H. somnus suspended in saline, the amount of pneumonia estimated and an effective dose determined by probit analysis. In addition, the clinical signs, body temperatures, gross and histologic lesions, peripheral blood parameters (leukogram, fibrinogen) and serologic response to H. somnus were determined and compared to those reported for naturally occurring H. somnus pneumonia and previous H. somnus experimental infections. Nasal shedding of H. somnus was monitored, and five non-infected control calves were housed with infected calves to gain information on possible routes of H. somnus transmission.

The second experiment was also designed to test two hypotheses:

1. Pulmonary inflammation induced by intratracheal exposure to saline suspended H. somnus follows a sequence of acute fluid, fibrin and cellular exudation from alveolar capillaries and bronchiolar mucosa to cellular exudation with early domination by neutrophils and macrophage domination later.

2. Over time neutrophilic exudates dominate the bronchiolar reaction while alveolar exudation progresses from neutrophil to macrophage dominance.

To test these hypotheses, 20 one to five-week-old

calves were infected intratracheally with 2.0×10^{10} H. somnus and examined over time to determine the sequence of events leading to the pneumonic lesions. Groups of 5 calves each were examined at 1, 6, 24 and 72 hours post-exposure. Non-infected controls were examined at 6 and 72 hours. Light microscopic examination of the changes was augmented by the use of both scanning and transmission electron microscopy. Grading of the bronchiolar and alveolar cellular components of the exudates was performed and these scores compared between time groups to test whether bronchiolar and alveolar cellular exudation occurred in a similar manner. In addition, the extent and severity of the pneumonia was scored and infected groups compared to controls to determine if the pneumonia was induced by the saline without bacteria.

The third experiment tested the hypothesis that H. somnus selectively adheres to bronchiolar epithelium in preference to alveolar epithelium. In this experiment, one possible explanation for the persistent purulent bronchiolitis observed in natural and experimental H. somnus pneumonia was examined by comparing bronchiolar mucosal association of H. somnus to alveolar association of H. somnus in an in-vitro lung explant system utilizing scanning electron microscopy.

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CHAPTER TWO

A MODEL OF *HAEMOPHILUS SOMNUS* PNEUMONIA IN CALVES

ABSTRACT

Fifteen 1-4 week-old, male dairy calves were exposed intratracheally to Haemophilus somnus (strain ISU 156-83) suspended in phosphate buffered saline at dosages ranging from 0.8×10^7 to 2.0×10^{10} colony forming units. Five other calves received sterile phosphate buffered saline without bacteria, also given intratracheally. All calves were kelled and examined at 72 hours post-exposure except for two calves killed at 6 hours in-extremis. Fourteen of the 15 calves exposed to H. somnus developed greyish cranio-ventral pneumonia with gross or microscopic foci of necrosis. Haemophilus somnus was isolated from the lungs of 7 of 10 calves receiving 1.0 to 20.0×10^9 H. somnus, 1 of 5 receiving 1.0 to 11×10^7 and none of the control calves. There was significant regression ($p = 0.01$) of the bacterial dose on the amount of pneumonia which developed. Calves receiving higher numbers of bacteria developed more extensive pneumonia. The 50% effective dose (ED_{50}) of H. somnus for the development of pneumonia was 1.3×10^9.

Intense foci of bronchiolar and alveolar necrosis were surrounded by regions with milder neutrophilic to fibrino-purulent bronchiolitis and mixed histiocytic-neutrophilic alveolar exudates. Vasculitis was present only in these necrotic foci. Alveoli surrounding necrotic bronchioles generally contained deeply eosinophilic fibrin masses. The gross and microscopic lesions seen in these experimentally exposed calves were similar to those described in naturally

occurring H. somnus pneumonia. Control calves did not develop lesions. These data indicate that experimental H. somnus pneumonia can be induced using bacterial dosages of 10^9 organisms given intratracheally.

INTRODUCTION

Although Haemophilus somnus has been commonly isolated from the pneumonic lungs of cattle, [1,2,22,23,29,39,48,51,59] few experimentally infected cattle have developed pneumonia typical of the naturally occurring disease. [6,11,14,27,31,34,36,40,43] Without a reproducible model of the pneumonic disease, little can be learned of the pathogenesis of the pneumonic process induced by H. somnus.

Hemorrhagic interstitial pneumonia with pulmonary vasculitis and thrombosis resulted from intravenous injection of cattle with H. somnus. [11,34] Intranasal instillation of H. somnus produced pneumonia in only one of 52 calves. [6,11,27,31,34,36,43] Intratracheal exposure to H. somnus suspended in egg yolk or 'E' medium resulted in pneumonia in 11 of 11 calves. [11,14,40] The effect of intratracheal exposure to similar amounts of egg yolk of 'E' medium has not been reported. Haemophilus somnus was recovered from the pneumonic lungs of 6 of the 11 calves, but other bacteria (Pasteurella multocida, P. hemolytica and Corynebacterium pyogenes) were also isolated from the pneumonic lungs of 8 of the 11 calves. Only 3 examples of experimentally induced H. somnus pneumonia, uncomplicated with other bacterial infections, have been published. [11,14,40] Lesions of purulent bronchopneumonia with multiple abscesses were produced in calves exposed intratracheally to H. somnus. [11,14,40] Others have reported producing pneumonia in calves exposed intratracheally to H. somnus,

but details of their studies have not been published. [24, 38] No attempts to determine a dose response of H. somnus for pneumonia production have been reported.

This paper reports the production of suppurative bronchopneumonia in 14 of 15 calves exposed intratracheally to H. somnus suspended in phosphate buffered saline. Haemophilus somnus was reisolated from the lungs of 8 of the 14 calves with pneumonia, and the pneumonia was more extensive and more severe in calves given higher numbers of organisms.

METHODS AND MATERIALS

BACTERIAL INOCULUM. The bacterium Haemophilus somnus (strain ISU 156-83) used in these experiments was originally isolated at the Iowa State University Veterinary Diagnostic Laboratory from the brain of a calf with lesions of infectious embolic meningoencephalitis (ITEME). In a pilot project, this isolate produced more extensive pneumonia in dexamethasone-pretreated calves than did other H. somnus isolates from various sources including several isolates from pneumonias. This organism was passaged several times in calves by intratracheal exposure, grown for 18 to 24 hours on brain heart infusion agar (Difco Laboratories, Detroit, MI) supplemented with 0.5% yeast extract and 10% bovine blood (BHI-Y-BAP), washed from the plates with brain heart infusion broth supplemented with 0.5% yeast extract and 5% fetal calf serum, divided into

1-2 ml aliquots and frozen at -70 C for later use.

The isolate was identified as H. somnus based on the following characteristics [10,19,21,26,27,30,46,49,59]: 1) small Gram-negative pleomorphic coccobacillus shape, 2) growth on or in supplemented media in microaerophilic environments containing 5-10% CO_2 at 37 C, 3) no growth in aerobic environments, 4) production of oxidase and the weak formation of indol, 5) weak fermentation of glucose, mannitol, maltose, trehalose and xylose and no fermentation of lactose, inositol, sorbitol, raffinose and salicin in liquid media supplemented with 5% fetal calf serum and incubated in 10% CO_2 at 37 C for 48 hours, 6) weak acidification of litmus milk and 7) agglutination by bovine serum containing H. somnus antibody.

EXPERIMENTAL GROUPS AND ANIMALS. Twenty 1-4 week-old calves weighing from 45 to 100 kg were purchased from three farms with no clinical history of H. somnus infection or of H. somnus vaccination. All were Holstein bull calves. Calves did not have serum complement-fixing antibodies to H. somnus and were not shedding H. somnus in nasal secretions or Salmonella spp. in their feces.

Calves were randomly assigned to 1 of 4 treatment groups of 5 calves each (Table 2-1). Group A was challenged with 0.8 to 2.0 X 10^{10} H. somnus suspended in 20 ml of phosphate buffered saline, group B received 0.8 to 2.0 X 10^{9} and group C received 0.8 to 11 X 10^{7} H. somnus. The

TABLE 2-1. Experimental groups of calves exposed intratracheally to Haemophilus somnus. Dosage, percentage pneumonia and isolation of H. somnus from lungs and nasal swabs.

Calf #	H. somnus Dosage	Percent Pneumonia	H. somnus isolated from lung	H. somnus from nasal swabs
Group A				
952	$2.0X10^{10}$	22	+	-
969	$1.3X10^{10}$	17	+	-
053	$1.3X10^{10}$	10	+	-
988	$1.1X10^{10}$	8	+	-
057	$0.8X10^{10}$	17	+	+
Group B				
783	$2.0X10^{9}$	6	+	+
058	$1.3X10^{9}$	2	-	+
981	$1.1X10^{9}$	8	+	+
339	$1.1X10^{9}$	21	-	-
987	$0.8X10^{9}$	3	-	-
Group C				
958	$1.1X10^{8}$	1	+	-
336	$1.1X10^{8}$	0	-	+
973	$0.8X10^{8}$	2	-	-
966	$2.0X10^{7}$	3	-	-
975	$0.8X10^{7}$	1	-	-
Controls				
055	none	0	-	-
982	none	0	-	-
335	none	0	-	-
338	none	0	-	-
313	none	0	-	-

fourth group received 20 ml of phosphate buffered saline without H. somnus and was kept in the same pens with H. somnus exposed calves.

All calves were housed in indoor pens at 15-22 C and fed a diet of pasteurized whole milk and free-choice alfalfa hay. No antibiotics were included in their rations. Calves were given 1 dose of a rota and corona virus vaccine (Norden Laboratories, Lincoln, NB).

PREPARATION OF INOCULUM. A frozen ampule of Haemophilus somnus (ISU 156-83) suspended in brain heart infusion broth was thawed and 2-3 drops placed on each of 6 to 8 BHI-Y-BAP plates. The H. somnus suspension was spread evenly over the surface of the agar with sterile cotton-tipped swabs and the plates incubated for 18 hours at 37 C in microaerophilic atmospheres containing 10% CO_2. The bacterial growth on the plates was checked for purity and tentatively identified as H. somnus based on morphologic and growth characteristics. The six plates with the heaviest growth of H. somnus were each flooded with 6 ml of sterile phosphate buffered saline and the bacterial growth gently scraped from the surfaces with glass spatulas. The bacterial suspensions were pooled, diluted to 50 ml and mixed for 30 seconds. The optical density of this suspension was read in a 12 mm diameter cuvette in a spectrophotometer (Coleman Jr II, Coleman Instruments, Maywood, IL) at 400 nm wavelength. This preparation was

used for exposing the calves in group A (the high dose group). Five ml of this suspension was added to 45 ml of sterile phosphate buffered saline and this diluted suspension used for the group B calves. A further tenfold dilution was made to prepare the inocula for group C. Once dilutions were made, 1 ml of fetal calf serum was added to each 50 ml of inoculum. The number of viable H. somnus in the inocula were estimated by making tenfold serial dilutions of an aliquot, inoculating BHI-Y-BAP plates with 0.1 ml of the 10^8 to 10^{12} dilutions and counting H. somnus colonies on all plates containing between 50 and 250 colonies.

CALF EXPOSURE PROCEDURE. A sterile 1 1/2 inch 12g needle was inserted through the skin of the ventral neck into the lumen of the trachea 3 to 5 cm below the larynx. A sterile 55 cm #5 Fr polypropylene urinary catheter (Sovereign, Monoject, Sherwood Medical, St. Louis, MO) with 2 oval tip openings 1 X 2 mm in diameter was passed through the needle into the trachea to a depth of 30 cm. Calves responded to the insertion of the catheter with frequent hoarse coughing. Twenty ml of the prepared inoculum was injected through the catheter while slowly withdrawing the catheter 6 to 8 cm. The catheter was flushed with 50 ml of air and 20 ml of sterile phosphate buffered saline before withdrawing the catheter and the needle from the trachea. Control calves received sterile phosphate buffered saline

containing 2% fetal calf serum without bacteria, followed by air and sterile phosphate buffered saline flushes in an identical manner to H. somnus exposed calves.

DATA COLLECTION. The calves were observed at 2, 12, 24, 48 and 72 hours following exposure and body temperatures, respiratory rates (while standing at rest), and breathing patterns recorded. In addition, the lungs of all calves were auscultated, and blood samples were collected from the jugular vein into tubes containing EDTA anticoagulant. Leukograms, hemoglobin concentrations, packed cell volumes, plasma protein values and plasma fibrinogen levels were determined by standard methods.

Serum was collected from each calf prior to exposure and at 72 hours post-exposure. Antibody titers to H. somnus were determined by the previously described complement fixation test (CFT) [7] and microscopic agglutination test (MAT). [8]

Nasal swabs were taken from the right nostril of each calf at 24 hour intervals beginning 24 hours prior to exposure and continuing to 72 hours post-exposure. Sterile cotton-tipped swabs were slid through a 12 cm long sterile plastic speculum which had been inserted 3 to 4 cm into the nostril. Swabs were then plated on 5% bovine blood agar and BHI-Y-BAP plates and incubated at 37 C in aerobic and 10% CO_2 environments, respectively, for 24-48 hours. The shape and Gram-staining characteristics of representative

colonies of bacterial growth were examined. All Gram-positive cocci were classified as such without further identification procedures. Gram-negative rods were identified by standard methods. All suspected H. somnus isolates were identified by the criteria listed above.

Nasal swabs taken 24 hours prior to exposure were also streaked on and swirled in Mycoplasma agar and broth (GIBCO Laboratories, Madison, WI). Suspicious mycoplasmal growth was identified by direct fluorescent antibody tests done on agar cubes containing suspect colonies.

NECROPSY PROCEDURES. After euthanasia by electrocution, the thorax and abdomen were opened and the lungs examined. The location, severity and character of the pneumonic changes were recorded on a standard size drawing of four different views of the lung (Figure 2-1). The caudal segments of the left and right cranial lobes were removed and inflated with 10% neutral buffered formalin at 28-30 cm pressure. The right middle lobe was inflated with cold 2.5% glutaraldehyde (Ladd Research Industries, Burlington, VT) buffered with 0.1M sodium cacodylate infused into the lobar bronchus via gravity flow with the level of the fixative maintained 28 to 30 cm above the opening of the bronchus. Cross sections of lung from other lobes were taken 2 cm from the tracheal-bronchial junction and fixed by submersion in 10% buffered formalin without inflation.

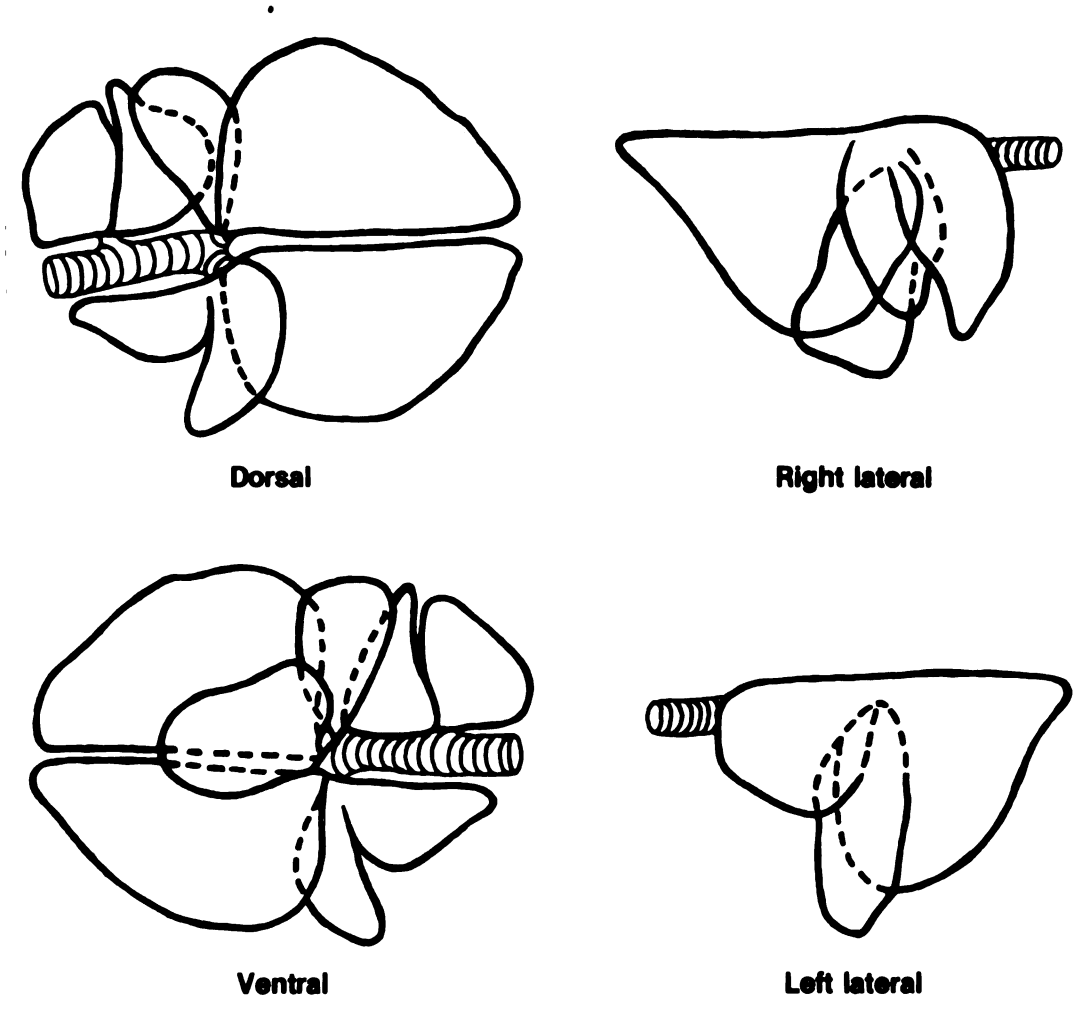

Figure 2-1. Standard drawings of bovine lung used to record location of pneumonia.

The proximal portions of all lung lobes not inflated with fixative were collected in sterile plastic bags and cultured for bacteria and mycoplasma as previously described for nasal swabs.

In addition to lung, cerebrospinal fluid, tracheobronchial lymph nodes, trachea, kidney, liver, urine, joint fluid, ileum and mesenteric lymph nodes were cultured for bacteria. Swabs of these specimens were streaked on both 5% BAP and BHI-Y-BAP plates and these plates incubated at 37 C in both aerobic and microaerophilic (10% CO_2) environments. Ileum and mesenteric lymph nodes were also incubated for 24 hours in sodium selenite broth and subsequently plated on brilliant green agar.

Lung, trachea and tracheobronchial lymph nodes were examined for the presence of bovine respiratory syncytial virus, infectious bovine rhinotracheitis virus, bovine virus diarrhea virus and parainfluenza-3 virus by both direct fluorescent antibody examination of frozen sections and by inoculating cell cultures. Cell cultures were examined at 7 day intervals for cytopathic effects and also examined by direct fluorescent antibody techniques for the presence of viral growth.

In addition to lung sections, brain, liver, kidney, trachea, nasal turbinate, tracheobronchial lymph node, ileum and colon were fixed in 10% buffered formalin, dehydrated, embedded in paraffin, sectioned at 5 to 6 micrometers, mounted on glass slides, stained with hematoxylin

and eosin, covered with glass coverslips and examined by light microscopy. Glutaraldehyde fixed lung was post fixed with osmium tetroxide, dehydrated, embedded in resin, sectioned at 1 to 2 micrometers, stained with toluidine blue, and examined by light microscopy.

ESTIMATING PERCENTAGE OF PNEUMONIA. The lung drawings (Figure 2-1) from each calf were overlain with a grid of 0.5 cm squares. The number of squares with pneumonia in each of the three different views of the same lung lobe were totaled and divided by the number of squares in that lobe to determine percent involvement. The lobar contribution of pneumonia to the entire lung was averaged from the three different views (dorsal, ventral, lateral) and the percentage of pneumonia weighted, based on each lobes contribution to the total lung surface. For example, if 30% of the accessory lobe was pneumonic (the accessory lobe contributes approximately 4% to total lung volume), the weighted contribution of that lobe to the percent pneumonia in the calf would be 30% X 4 or 1.2%. The weighted contributions of all lobes were added to determine total percent pneumonia. Using this system, the lobar contributions were right cranial 15%, right middle 8%, right caudal 29%, accessory 4%, left cranial 16% and left caudal 28%.

ANALYSIS OF DATA. The effective dose (ED_{50}) for pneumonia produced by H. somnus was determined using probit analysis of dosage versus response (percent pneumonia). [16,35] Percent pneumonia data were transformed using an arcsine square root conversion prior to probit analysis. The leukograms, body temperatures, respiratory rates, plasma fibrinogen levels and plasma protein to fibrinogen ratios were compared to pre-exposure values using a randomized complete block design analyzed non-parametrically using Friedman's test. [53] Comparisons of respiratory rates and leukograms between sampling times were also performed using a paired design and Wilcoxon's Signed Rank test. [53] Comparison of the amount of pneumonia between treatment groups was done using the Kruskal-Wallis test and individual pairs of treatment groups were compared with the Wilcoxon-Mann-Whitney Two-Sample test (Rank Sum test). [53]

RESULTS

CLINICAL SIGNS, CLINICAL PATHOLOGY AND SEROLOGY.

Nine of the ten calves in groups A and B developed hypernea (Figure 2-2) and dyspnea within 2 hours post-exposure and 5 had mild leukopenia (Figure 2-3) with relative neutropenia. Body temperatures did not vary significantly from pre-exposure values in any treatment group at any time post-exposure (Figure 2-4).

Two calves in group A (the high dose group) became

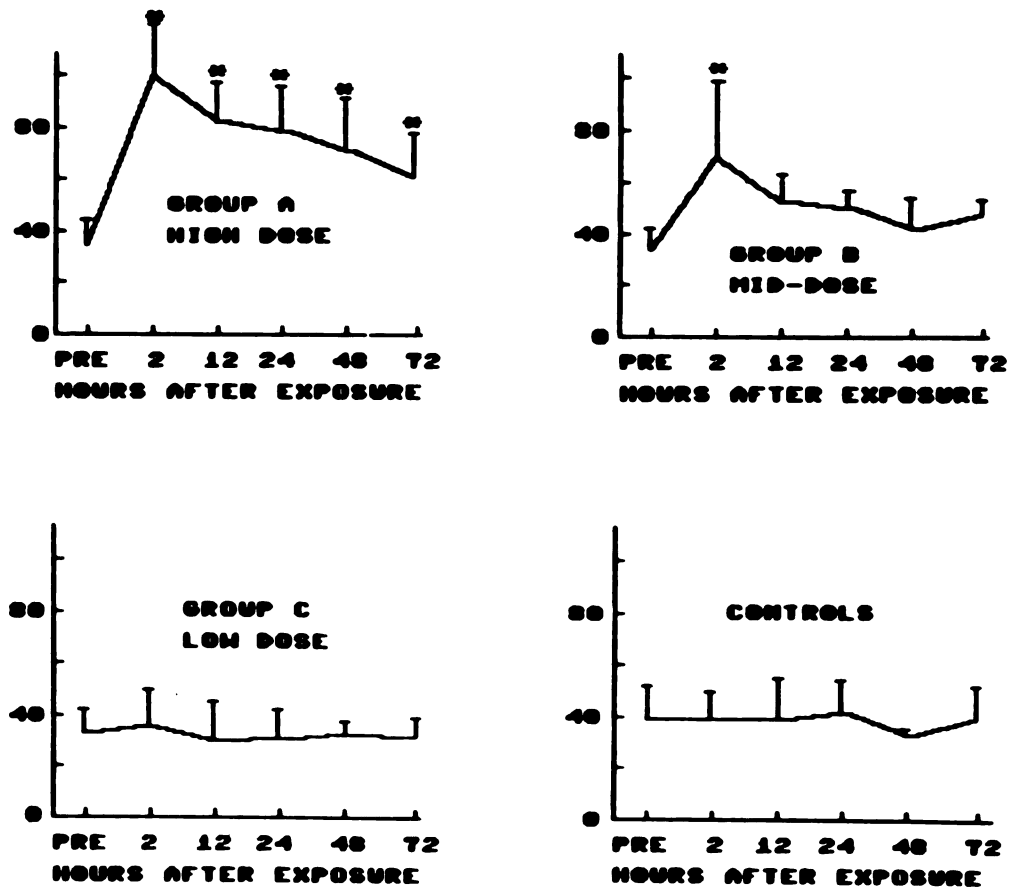

Figure 2-2. Mean respiratory rates (breaths per minute) in calves exposed intratracheally to Haemophilus somnus. * = significant difference (p = 0.05) from pre-exposure mean.

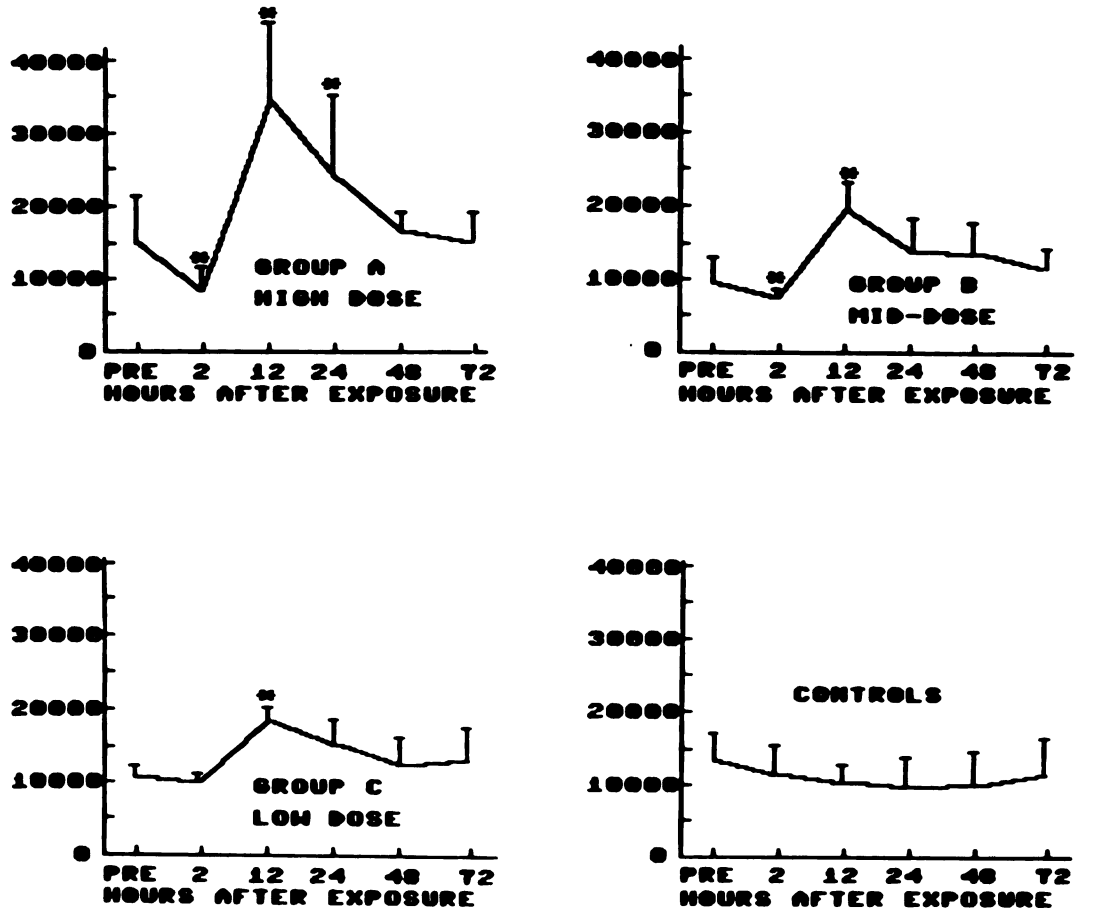

Figure 2-3. Mean leukocyte numbers per cubic mm in peripheral blood of calves exposed intratracheally to Haemophilus somnus. * = significant difference (p = 0.05) from pre-exposure mean.

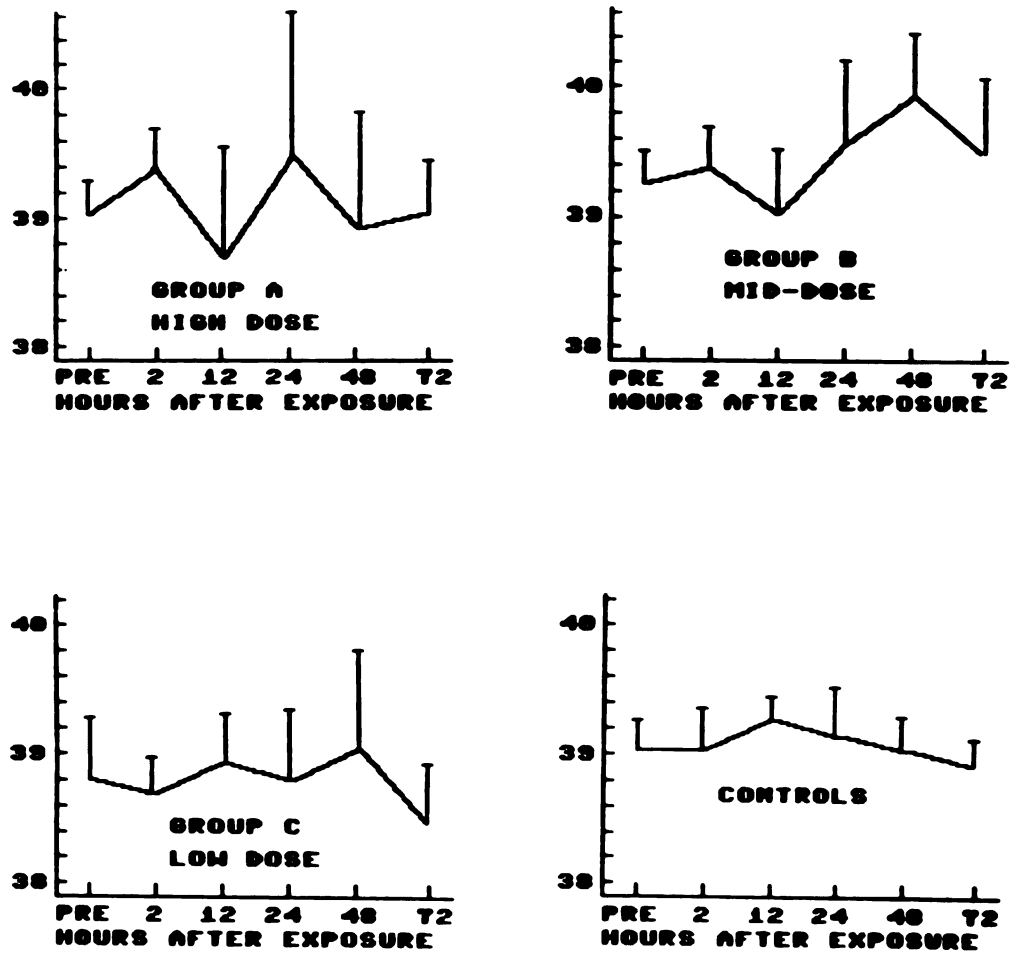

Figure 2-4. Mean rectal temperatures (degrees centigrade) in calves exposed intratracheally to Haemopilus somnus.

recumbent and unable to rise by 6 hours post-exposure and were euthanatized. Moist rattles were auscultated in calves in groups A and B, 2 to 6 hours post-exposure but were not heard at 24, 48 or 72 hours post-exposure. Occasional coughing was noted in group A calves. Coughing was not noted in other groups.

The severe dyspnea, hyperpnea and depression observed within a few hours of exposure, dissipated by 6 to 12 hours. However, respiratory frequency remained greater (figure 2-2) than pre-exposure rates ($p = 0.05$) in H. somnus exposed calves in group A throughout the experiment although dyspnea was not observed after 12 hours.

Leukocyte numbers in peripheral blood from H. somnus exposed calves reached their maximum at 12 to 24 hours post-exposure and then declined to pre-exposure values in calves by 72 hours post-exposure (Figure 2-3). As a group, the leukocyte counts of calves receiving H. somnus were significantly increased over pre-exposure values ($p = 0.05$) while leukocyte counts in control calves decreased. Increased leukocyte numbers were the result of neutrophilia with high numbers of non-segmented neutrophils and metamyelocytes appearing in the peripheral blood as early as 12 hours post-exposure. Calves in group C (low dose group) did not develop respiratory signs or leukopenia at 2-6 hours post-exposure, but leukocyte counts increased in these calves by 12 hours post-exposure and returned to near pre-exposure numbers in most by 48 hours (Figure 2-3).

Plasma fibrinogen in calves exposed to H. somnus increased by 24 hours, but these increases were not significantly different from pre-exposure values ($p = 0.05$) until 48 to 72 hours (Figure 2-5). Control calves had fibrinogen levels as great as infected calves, but their post-exposure values did not differ significantly from pre-exposure levels. Declines in the plasma protein/fibrinogen ratios, over time, were not statistically significant.

None of the calves developed serum complement fixing (CFT) antibody titers exceeding 1:4 (Table 2-2) by 72 hours post-exposure but all calves had microscopic agglutinating (MAT) antibody titers ranging from 1:16 to 1:4096. Fourfold or greater MAT titer rises occurred by 72 hours post-exposure in 2 infected calves and one control calf. Two control calves had greater than fourfold MAT titer decreases in that same time. No fourfold CFT antibody changes were detected in any calves 72 hours post-exposure.

MICROBIOLOGIC RESULTS. Haemophilus somnus was isolated from pneumonic lung lobes of all 5 calves in group A, 2 of 5 in group B and 1 in group C (Table 2-1). No Haemophilus somnus were isolated from the lungs of contact control calves. Haemophilus somnus was also isolated in pure populations from at least one pneumonic lobe of these 8

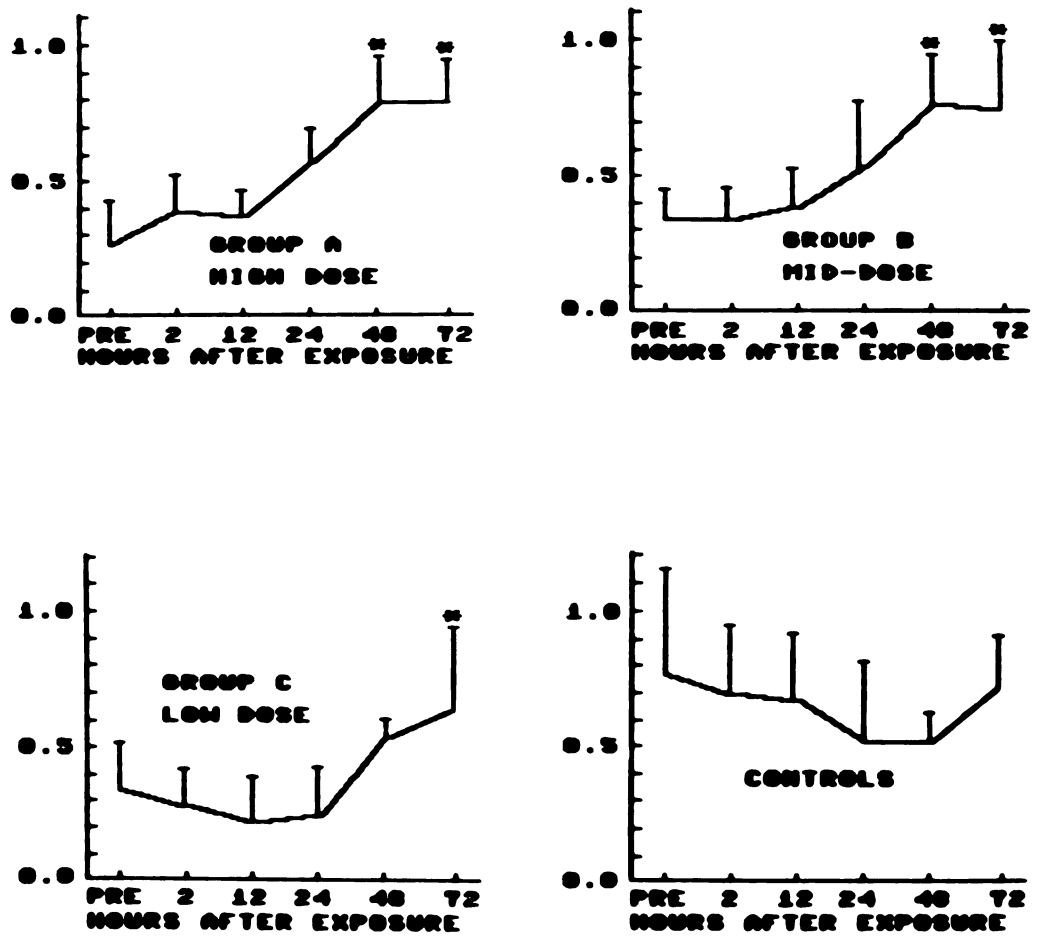

Figure 2-5. Mean plasma fibrinogen (mg/dl) in calves exposed intratracheally to Haemophilus somnus. * = significant difference (p = 0.05) from pre-exposure mean.

Table 2-2. Microscopic agglutination test (MAT) and complement fixation test (CFT) antibody titers in calves prior to exposure and 72 hours after intratracheal exposure to Haemophilus somnus.

Calf #	MAT titers pre-exp.	MAT titers 72 hrs	CFT titers pre-exp	CFT titers 72 hrs
Group A				
952	16	16	neg.	neg.
969	64	64	neg.	neg.
053	128	---	neg.	neg.
988	512	---	neg.	neg.
057	256	1024	neg.	neg.
Group B				
783	128	256	neg.	neg.
058	16	512	neg.	neg.
981	256	512	neg.	neg.
339	256	512	neg.	neg.
987	128	128	neg.	neg.
Group C				
958	128	128	4	4
336	16	16	neg.	neg.
973	32	32	neg.	neg.
966	16	32	neg.	neg.
975	64	64	neg.	neg.
Controls				
055	1024	128	neg.	neg.
982	1024	4096	4	neg.
335	32	64	neg.	neg.
338	128	32	neg.	neg.
313	512	1024	neg.	neg.

calves although P. multocida was also isolated from other lobes of 4 calves (952, 969, 057 and 966). In addition, H. somnus was isolated from the tracheas of 3, the tracheobronchial lymph node of 1, and inflamed subcutaneous tissues of the neck of one. Pasteurella hemolytica was not isolated from the lungs of any of the calves.

Haemophilus somnus was also isolated from the nasal swabs of 4 of 15 infected calves and none of the controls (Table 2-1). Pasteurella multocida was isolated at least once from the nasal swabs of 8 of 15 H. somnus infected calves and 4 of 5 controls. Gram-positive cocci were isolated from the nasal swabs of 13 of 15 H. somnus infected calves and 5 of 5 controls. Other isolates from the nasal swabs included P. hemolytica (2 calves, E. coli (6 calves) and M. bovirhinis (5 calves).

No viral agents were isolated or demonstrated from lung, trachea or tracheobronchial lymph nodes of any calves. Likewise, no H. somnus was isolated from cerebrospinal fluid, urine, kidney, liver, mesenteric lymph nodes, ileum or joint fluid of any calf.

GROSS LESIONS.

GROUP A. Two calves in group A (high dose) were killed in-extremis 6 hours post-exposure and had interlobular edema with reddish-grey consolidation of the lung. Only H. somnus was isolated from the lungs of these two calves. The 3 remaining group A calves when examined at 72 hours PI

had greyish consolidation of multiple lung lobes with lesions primarily in cranial lobes (Figure 2-6) involving from 17 to 22% of the lung (Table 2-1). Multifocal necrosis was common near the hilus of affected lobes with scattered necrotic foci in remaining regions (Figure 2-7). Mediastinal and tracheobronchial lymph nodes were edematous.

GROUP B. Except for one calf (#339), group B calves had less extensive pneumonia than group A (Table 2-1) ranging from 2 to 8%. Calf #339 had 21% pneumonia. Multifocal necrosis was observed grossly in the lungs of only one calf (#783).

GROUP C. Calves receiving the lowest dose of H. somnus had no lesions or only small focal pneumonic changes (Table 2-1). Haemophilus somnus was isolated from only 1 of these calves, and this isolation was from a single grossly observable focus of necrosis.

Groups A and B had significantly more pneumonia ($p = 0.05$) than did group C or the controls. Group A also had significantly more pneumonia than group B. The ED_{50} for pneumonia production as determined by probit analysis was 1.3×10^9 with a 95% confidence interval from 6.0×10^7 to 2.7×10^{10}. There was significant ($p = 0.01$) regression of bacterial dose on the amount of pneumonia produced.

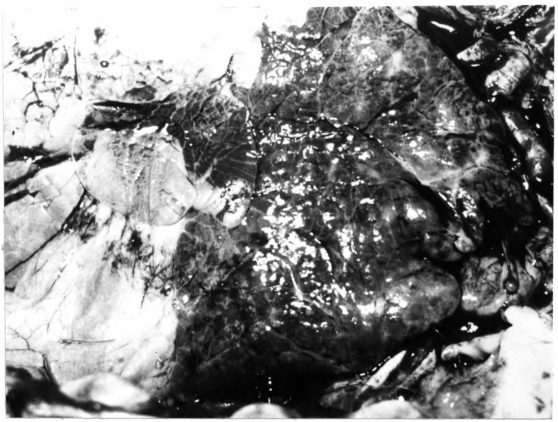

Figure 2-6. Photograph of cranial ventral pneumonic consolidation in the right lung of a calf exposed intratracheally to Haemophilus somnus 72 hours previously.

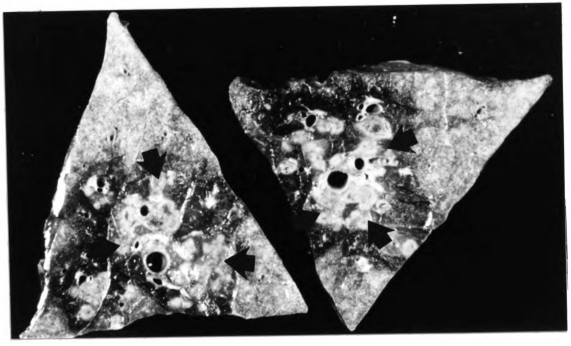

Figure 2-7. Photograph of multiple foci of necrosis (arrows) in the lung of a calf exposed intratracheally to Haemophilus somnus 72 hours previously.

MICROSCOPIC LESIONS. Three relatively distinct reactions were observed in the lungs. These reactions were classified as mild, moderate and severe inflammation and the characteristic changes in each of these reactions are described below.

MILD INFLAMMATION OF THE LUNGS. Mild inflammation of the lungs was characterized by multifocal neutrophilic bronchiolitis with mild pneumonia. Increased numbers of neutrophils and large macrophages in varying ratios were present in bronchioles, alveoli immediately adjacent to affected bronchioles and in randomly scattered alveoli. Occasionally condensed fibrinous exudate accompanied the alveolar cellular exudation (Figure 2-8). Alveolar walls were only slightly thickened with increased numbers of inflammatory cells in the walls and swollen pneumocytes and macrophages on the luminal surfaces. Neutrophils were increased in bronchiolar submucosal capillaries and venules and were present between bronchiolar epithelial cells. A few bronchi contained fibrinocellular exudates in the lumens or had inflammatory cells in the bronchial mucosa.

In sections of lung fixed without inflation, these mildly affected areas were typically atelectatic. There was no dilatation of lymphatic channels, vascular degeneration or inflammation seen in these areas.

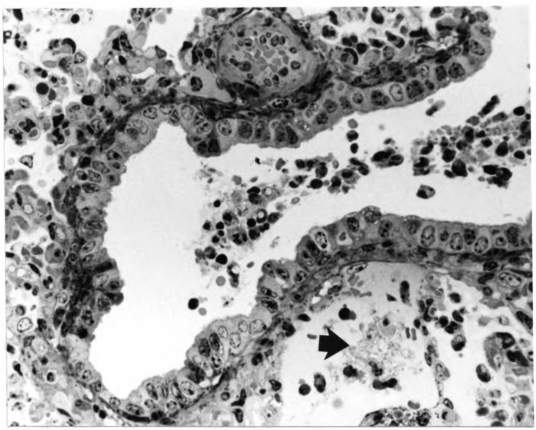

Figure 2-8. Photomicrograph of a lung with mild inflammation. A few neutrophils and macrophages are present in bronchioles and alveoli. Small fibrin masses are present in scattered alveoli (arrow). (toluidine blue). X 768.

MODERATE INFLAMMATION OF THE LUNGS. Moderate inflammation of the lungs was characterized by extensive neutrophilic bronchiolitis usually accompanied by histiocytic pneumonia. There were regions of lung in which bronchioles were filled and distended with neutrophils and lesser numbers of macrophages (Figure 2-9). Numerous inflammatory cells (primarily neutrophils) were also present in bronchiolar walls and in submucosal vessels. Neutrophils often lined the bronchiolar surfaces in a "pavementing" arrangement. Only patchy areas of bronchiolar epithelium appeared necrotic. Bronchi were either unaffected or contained scant cellular exudates. Alveoli immediately surrounding affected bronchioles were distended with inflammatory exudates containing primarily macrophages and fibrin with lesser numbers of neutrophils and erythrocytes. A few areas of alveolar inflammation without prominent bronchiolar involvement were seen. A transition of inflammatory cell types from predominantly neutrophils to primarily macrophages was present in longitudinal sections of terminal bronchioles and alveolar ducts (Figure 2-9). Acellular fibrinous exudates in alveoli were not consistently found and when present tended to be multifocal. In lungs fixed without inflation, the alveoli had a tendency to collapse making the alveolar inflammation more difficult to observe and the bronchiolitis more prominent.

Alveolar walls were mildly thickened with inflammatory cells and swollen pneumocytes. Necrosis of alveolar walls

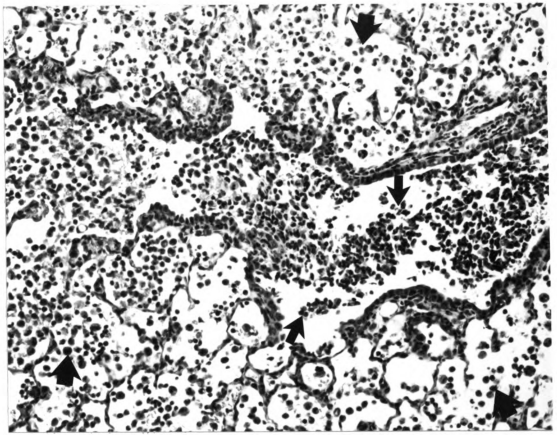

Figure 2-9. Photomicrograph of a moderately inflamed lung. Macrophages fill alveoli (large arrows) while bronchioles contain mixed inflammatory cell exudates with neutrophils predominating (small arrows). HE. X 445.

or vascular inflammation and degeneration were not present in moderately inflammed regions of lung. Interlobular septal and perivascular lymphatics were occasionally dilated with fibrinous and fibrinocellular exudates.

SEVERE INFLAMMATION OF THE LUNGS. The main distinguishing feature of lesions classified as severe was necrosis, including necrosis of the inflammatory cells that packed and distended bronchioles and alveoli (Figure 2-10). Degenerating inflammatory cells occasionally formed streaming patterns. Necrosis of the bronchiolar mucosa, transmural bronchiolar wall necrosis, and necrosis of alveolar walls accompanied the necrotic inflammatory exudates. Interlobular septal lymphatics surrounding lobules with the severe reactions were distended with fibrinous and fibrinocellular exudates. In most lobules, the alveoli immediately peripheral to the most severely affected bronchioles were filled with deeply basophilic inflammatory cells often with bacteria present. In other severely affected lobules, the intense cellular filling of alveoli was more peripheral to the bronchioles with the centrally located alveoli filled with fibrinous fluid and erythrocytes (Figure 2-10).

Arterioles, veins and capillaries within these necrotic foci frequently contained thrombi. Inflammatory cell infiltration into the adventitia and media of arterioles and arteries was common.

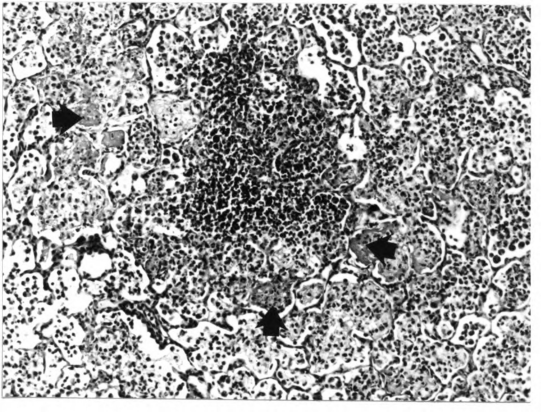

Figure 2-10. Photomicrograph of a lung with severe inflammation. Focal necrosis is present in the center of the photomicrograph surrounded by dense fibrin (arrows) filling alveoli. HE. X 325.

Although the intense foci of inflammation classified as severe, were found in all groups of H. somnus exposed calves, the number, size, and extent of the foci were less in lungs exposed to smaller bacterial numbers. Necrotic foci were found microscopically in 3 of 3 group A calves examined 72 hours post-exposure, 4 of 5 group B calves and 3 of 5 calves in group C. Necrotic foci were seen grossly in only 1 of 5 group C calves.

Moderate inflammation predominated in the lungs of calves in groups A and B with these moderate changes bordering areas of more severe pulmonary necrosis. Calves in group C had predominantly mild inflammation with only a few foci of moderate and severe inflammation. Control calves had no gross or microscopic lesions.

Tracheobronchial lymph nodes were edematous and contained increased numbers of neutrophils in the medullary, cortical and subcapsular sinusoids. These inflammatory reactions were most intense in group A calves and were absent in 4 of 5 group C calves and the controls.

The tracheas of 10 calves, including 8 exposed and 2 control calves, had increased numbers of neutrophils, macrophages and occasional plasma cells in the superficial submucosa with scattered neutrophils in the tracheal epithelium. The right nasal turbinates had increased numbers of inflammatory cells in the submucosa of both control and exposed calves. Erosions and ulcerations were present in the mucosa of the right turbinate of 6 calves, 4 exposed

and 2 controls. These erosions and ulcers were present in the areas of repeated nasal swabbing.

Brains, hearts, livers and kidneys of all calves were normal.

DISCUSSION

Acute neutrophilic bronchopneumonia with many of the characteristics of naturally occurring H. somnus pneumonia was produced by intratracheally inoculating young calves with H. somnus bacteria suspended in sterile phosphate buffered saline. The extent of lung lesions produced was dose dependent with more extensive and more severe lesions in calves given the highest numbers of bacteria.

Clinical signs produced by intratracheal H. somnus inoculation were similar to those described by others. [11] Corboz and Pohlenz [11] observed severe depression and tachypnea within a few hours after intravenous and intratracheal inoculation of H. somnus. They attributed this response to endotoxemia, although they gave no further reasons for this opinion. Neutropenia and lung edema occur after intravenous endotoxin administration. [4] We also observed severe depression, tachypnea and neutropenia within 6 hours post-exposure especially in the group receiving the highest number of H. somnus (group A).

The onset of severe clinical signs within hours of exposure to H. somnus, corresponded to the occurrence of neutropenia. Neutropenia coupled with the neutrophilic

lung lesions suggest the changes in the peripheral blood leukogram are related to neutrophil migration into lung parenchyma and airways. The lack of prominent clinical signs after the first 12 hours following exposure to H. somnus, including inconsistent body temperature rises and a lack of coughing, is similar to many natural outbreaks of H. somnus pneumonia in which the first sign of herd infection is an animal death. [1]

The neutrophilic bronchiolitis and the peribronchiolar filling of alveoli with dense fibrinous and fibrinocellular exudates, common in the natural disease, [1,2,52] were present in these experimental infections. The severe necrotizing bronchiolitis observed in over one-half of the natural H. somnus pneumonias, [1] was common in regions with severe inflammation in this model. The necrotic foci seen in these calves may progress to abscess formation, a feature reported by others [40] especially in experimental infections. [11] Gross lesions of diffuse fibrinohemorrhagic pleuropneumonia typically associated with P. hemolytica pneumonia [49,63] were not seen in this model. "Streaming" inflammatory exudates commonly reported in P. hemolytica pneumonias [49,53,63] were seen only in the severly affected regions of these H. somnus infected calves.

An interstitial pneumonia in more caudal dorsal regions of the lungs was not observed in these experiments. A concurrent viral infection has been proposed as a possible

cause for these lesions frequently seen in naturally occurring H. somnus pneumonia.[1] Further experimentation is necessary to determine the interrelation of H. somnus with respiratory viruses. A brief report[39] has suggested that experimental infection with bovine respiratory syncytial virus allows induction of H. somnus pneumonia.

The location of lung lesions produced by intratracheal exposure to fluid suspended H. somnus were similar to the locations of lung lesions in the natural disease[1] with cranial and ventral aspects of the lung being primarily affected. Lobar localization in one of these experimental calves suggests that this exposure technique may occasionally deposit the majority of the inoculum in one lobar bronchus.

The accessory lobe in this model was also commonly affected. Although deposition of H. somnus in certain locations in preference to others may explain the location of lesions and would therefore be largely determined by gravitational effects on fluid flow within the bronchial tree, other explanations are possible. An alternate possibility explaining the higher incidence of lesions in the cranial and accessory lobes is the possible higher susceptibility to bacterial pneumonia of lobes with smaller parenchyma to surface ratios.[42,43] The tethering effects of interdependence is less in these lobes, possibly reducing the effectiveness of clearing airways plugged with inflammatory exudates.[42,43]

Of the three patterns of lung injury (mild, moderate and severe) observed in these experimental calves, the moderate inflammatory reactions most closely resembled the lesions of the naturally occurring disease. The necrotizing features of the reactions classified as severe are frequent in the natural disease, but the streaming inflammatory exudates are not. [1,2] Mild lesions were probably in a stage of resolution and healing. No H. somnus were isolated from lungs or lobes of lungs with only mild lesions.

Although vasculitis occurred in the lungs of many of these calves, the vasculitis was associated with severe inflammation and necrosis and appeared to be an extension of that reaction. Vasculitis in the regions of necrosis following intratracheal exposure to H. somnus has been reported by others. [11,21] Vasculitis did not appear as a lesion independent of the pneumonia. No evidence in other tissues was present to suggest that a bacteremia or septicemia developed. Haemophilus somnus septicemia is rarely reported [56] in calves as young as those used in this study. It is also uncommon to observe H. somnus septicemia and active H. somnus pneumonia in the same animal. [1,26,56]

The lack of pneumonic changes in control calves 72 hours after receiving intratracheal sterile phosphate buffered saline suggests that the sterile phosphate buffered saline carrier for the bacteria has no morphologic effect in the lungs of calves examined 72 hours later. Administration of small volumes of fluid similar to those

used here, into calf lungs, induced transient changes in arterial oxygen and arteriolar alveolar oxygen difference and mild neutrophil infiltration. [28,51]

In a previous study, this author examined calves exposed to H. somnus 12 to 14 days previously (Jackson, JA, Andrews, JJ and Hargis, J: unpublished data). Others [11] killed and examined calves 6 days post-exposure. In this study we chose to examine calves 72 hours post-exposure to observe more acute stages of H. somnus induced pneumonia than had been previously described and to minimize the frequent complication of isolating other bacteria such as Pasteurella spp. or Corynebacterium spp. from pneumonic calf lungs in experiments of longer duration. [11,14,41]

Although fibrinogen and plasma fibrinogen to plasma protein ratios have been suggested as sensitive indicators of inflammatory changes in cattle, [15] these tests were not of much value in predicting the presence of pneumonia in these young calves. Prior diarrheal disease may have elevated fibrinogen levels in both inoculated and control calves.

The MAT titers to H. somnus were considerably higher than the CFT titers in the same animal. Similar differences have been described in previous reports. [46] High pre-exposure MAT titers did not seem to prevent H. somnus pneumonia. The lack of significant CFT titers to H. somnus in animals with relatively high MAT titers suggest that the MAT test and the CFT test do not measure the same

response. The MAT may be overly sensitive in determining prior H. somnus exposure. No lesions or microbiologic evidence of H. somnus infection was detected in control calves although 3 of 5 had MAT titers of 1:512 or greater. The one H. somnus exposed calf with an MAT titer of 1:512 (calf 988, group A) developed severe clinical signs and was euthanatized in-extremis 6 hours post-exposure. Possibly high levels of circulating antibody may increase pneumonia development following pulmonary exposure to bacteria. [18] Microscopic agglutination test, ELISA, and CFT antibodies did not protect calves from the development of ITEME following intravenous challenge. [55,57]

Two animals in group A became recumbent and were euthanatized in-extremis. Acute lung edema and massive neutrophilic infiltration of bronchioles and alveoli characterized the lesions in these 2 calves. Similar lesions have been induced with intrabronchial instillation of activated complement in rats [25] and with E. coli endotoxin in sheep. [5] Neutrophils release oxygen derived radicals (hydroxyl radicals, superoxide anion and singlet oxygen), [58,59] peroxidases and proteases into the lung environment and these damage lung. [62] The chemotaxis of bovine neutrophils by H. somnus derived factors has not been investigated. Bovine neutrophils do not respond to bacterial derived formylated oligopeptides but do respond chemotactically to products of Gram-negative bacteria [17] therefore, it is likely that neutrophils directly respond

chemotactically to H. somnus.

Daily nasal swabbing in this experiment was associated with an increased rate of isolation of P. multocida from the nasal swabs of both H. somnus exposed and control calves. Although P. multocida was often isolated, increased isolation rates of P. multocida were not reported in older calves repeatedly nasal swabbed. [32] Damage to the nasal mucosa may enhance the colonization of P. multocida in the nasal passages as has been demonstrated in pigs. [45] The common presence of P. multocida in the upper respiratory tract of cattle, [32] its apparently rapid involvement in pneumonic lesions induced by H. somnus, coupled with the ability of P. multocida to grow more easily than H. somnus on artificial media, present both experimental and diagnostic complications when using conventional calves for producing H. somnus pneumonia. The use of gnotobiotic calves might also be considered in future experiments to reduce problems with concurrent Pasteurella spp. infection.

Nasal shedding of H. somnus occurred in only a few of the exposed calves and was only transient. The natural spreading of H. somnus by exposure of cattle to nasal secretions containing H. somnus has been suggested. [6] Haemophilus somnus may survive in nasal secretions for up to 70 days at 23.5 C. [13] These experiments did not suggest that H. somnus had any particular affinity for the nasal mucosa enabling it to colonize the nasal passages as has been suggested by in-vitro experiments. [61] Likewise, the

presence of Gram-positive cocci did not appear to enhance H. somnus nasal colonization as has been suggested by in-vitro experiments.[9] Rather, the low number of nasal shedders in these experimental calves resembled the low H. somnus shedding reported in naturally infected H. somnus herds.[12,37,48] Factors such as concurrent viral infections may be necessary for high numbers of calves to become nasal shedders of H. somnus.[12]

Subcutaneous abscesses at the site of intratracheal inoculation occurred in 2 H. somnus-exposed calves. Haemophilus somnus was isolated from one abscess. Since fluid resembling the inoculum was occasionally coughed out or appeared at the nostrils during the inoculation procedure, contamination of the tracheal puncture site with inoculum from the tracheal lumen may occur during exposure. Others have reported subcutaneous abscesses at sites of H. somnus inoculation into the trachea.[11,14,34,41] Perhaps only relatively low numbers of H. somnus in subcutaneous tissues are enough to produce an inflammatory reaction. This has not been studied.

The use of optical density measurement to estimate the number of viable H. somnus organisms in a saline suspension allowed the standardization of the exposure dosage of H. somnus organisms. Previous experimental inoculations of calves with H. somnus have used widely varied numbers of organisms. Because optical density is influenced by the numbers of suspended particles including bacteria and

dissolved colored substances, careful preparation of the inoculum is necessary to insure a standard dose. [33] Other workers have used spectrophotometric methods to estimate H. somnus numbers in suspensions. [3,35,36,38] Although we used wavelengths of 400, 540, 560, and 600 nanometers, a wavelength 400 nanometers was more sensitive to bacterial concentration changes than were the higher wavelengths used by others. [3,35,36,38] Haemophilus somnus suspended in saline in concentrations of 4-10 X 10^8 organisms/ml in 12 mm diameter cuvettes had optical densities between 0.4 to 0.8. Concentrations below 1.0 X 10^8 or above 4 X 10^9 were beyond the accurate scale on the spectrophotometer.

Although probit analysis has been used to predict dosages where mid-range lethal effects (LD_{50}) occur in H. somnus in-vitro infections, [36] this is the first report using probit analysis to predict mid-range non-lethal effects (effective dose-50 or ED_{50}) of H. somnus. Detecting changes between treated and non-treated or vaccinated versus non-vaccinated calves is necessary to evaluate the effectiveness of treatments or vaccination procedures for H. somnus pneumonia. Probit determination of ED_{50} offers a method of predicting the relationship between H. somnus dosage and effect and selecting a mid-range inoculation dosage. In this experiment, the loss of 2 calves in the high dose group (group A) at 6 hours post-exposure may have lowered the calculated ED_{50} because these 2 calves had less visible pneumonia than other calves in

group A.

Different researchers have produced pneumonia in cattle with varied numbers of H. somnus suspended in different materials. Dierks [14] produced pneumonia in 2 calves using intratracheal exposure to 10^7 H. somnus in egg yolk. Corboz and Pohlenz [38] used 1.9 to 11 X 10^8 H. somnus in egg yolk to produce pneumonia in 4 calves. Pritchard [41] used 5 to 14 X 10^9 H. somnus suspended in 'E' medium to produce pneumonia in 2 dairy calves. Groom [24] used 1 X 10^9 H. somnus to produce pneumonia in 5 calves. Gogolewski [20] used 10^7 to 10^9 H. somnus to produce pneumonia in an unspecified number of calves. Details of these last two experiments have not been published. Our determination of an ED_{50} for pneumonia production of 1.3 X 10^9 H. somnus suspended in saline is slightly higher than the dose of H. somnus suspended in egg yolk but is close to the dosage of H. somnus suspended in 'E' medium used to produce pneumonia. The inclusion of foreign material such as egg yolk in the inoculum may have a detrimental effect on phagocytes and the removal of H. somnus from the lungs. This may lower the necessary dose of H. somnus needed to produce pneumonia.

The calf model described herein produces many of the pneumonic lesions observed in naturally occurring outbreaks and offers the advantages of 1) predetermining the dosage and 2) suspending the inoculum in materials which do not damage lung or have undesirable effects on host defense

mechanisms.

The isolation of H. somnus from the pneumonic lesions following experimental exposure confirms that H. somnus is a respiratory pathogen of young calves. The method of pneumonic induction described in this paper provides a reproducible model for further study of the pathogenesis of the pneumonic aspects of this disease.

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CHAPTER THREE

SEQUENTIAL EVENTS IN *HAEMOPHILUS SOMNUS* PNEUMONIA OF CATTLE: HISTOLOGIC AND ULTRASTRUCTURAL STUDIES

ABSTRACT

The development of lung lesions was examined by light and transmission and scanning electron microscopy following intratracheal exposure of 20 calves to Haemophilus somnus. Neutrophilic exudation into alveoli and bronchioles began as early as 1 hour post-exposure and was extensive by 6 hours. Neutrophil efflux was from both alveolar capillaries and the bronchiolar submucosal vasculature. Neutrophils developed close associations with bronchiolar luminal surfaces and interdigitated with microvilli and cilia. Bronchiolar exudates were predominantly composed of neutrophils through 72 hours post-exposure while alveoli contained predominantly macrophages by 24 hours. The cellular composition of alveolar and bronchiolar exudates differed significantly at 6, 24, and 72 hours, and the differences increased with time. Necrosis of inflammatory cells and pulmonary tissues began as early as 24 hours post-exposure in regions with large numbers of bacteria. Vasculitis was present only in regions of necrosis. From these data it was suggested that the characteristic neutrophilic bronchiolitis, accompanied by mononuclear alveolar exudation, caused by H. somnus in bovine lungs results from a difference in the mechanisms of inflammation induced by mild injury in bronchioles when compared to alveoli. Severe injury, characterized by necrosis, is related to the continued presence of both bacteria and neutrophils in bronchiolar and alveolar locations.

INTRODUCTION

The lesions of naturally occurring Haemophilus somnus pneumonia differ from Pasteurella hemolytica pneumonia of cattle in two important ways. 1) Haemophilus somnus pneumonia is characterized by a bronchiolitis with less extensive alveolitis than P. hemolytica pneumonia while P. hemolytica pneumonia is primarily an alveolitis with minimal bronchiolar reaction.[17,48] 2) Lobular hemorrhage, inflammation and necrosis is far more severe and extensive in P. hemolytica pneumonia than in H. somnus pneumonia. "Streaming" necrotic inflammatory cells and fibrinous pleuritis are common with P. hemolytica pneumonia but are not with H. somnus.[1,2,17,48] The reasons for these differences are not understood.

Histologic lesions in the lungs of calves examined at 6 and 72 hours after intratracheal exposure to H. somnus (Andrews, JJ: thesis, chapter 2) suggest that the pulmonary lesions develop by acute fluid, fibrin and cellular efflux into bronchioles and alveoli possibly through submucosal post-capillary venules and alveolar wall capillaries.[56] Neutrophils predominated in the bronchiolar exudates of all calves examined 72 hours post-exposure while alveolar exudates were composed principally of macrophages. Only 2 animals were examined prior to 72 hours, and little understanding of the events leading to the pulmonary lesions could be ascertained. Because a neutrophilic to necrotic bronchiolitis is a characteristic feature of

naturally occurring H. somnus pneumonia, an understanding of the sequential events leading to this bronchiolitis is needed.

This paper reports the gross, microscopic and ultrastructural changes in 11 control calves and 20 calves exposed intratracheally to 2 X 10^{10} H. somnus. Calves were examined from 1 to 72 hours following exposure. Calves given H. somnus developed neutrophilic bronchiolar exudates by 6 hours post-exposure. Bronchiolar exudates remained predominantly neutrophilic, while alveolar exudates were composed of proteinaceous fluid and neutrophils early in the course of the disease and became predominantly composed of macrophages by 72 hours post-exposure.

METHODS AND MATERIALS

BACTERIAL INOCULUM: An isolate of H. somnus (ISU 156-83) previously used to produce pneumonia in calves was prepared as previously described (Andrews, JJ: thesis, chapter 2). The organisms were scraped from 18 hour culture plates and suspended in sterile phosphate buffered saline. The suspensions from multiple plates were pooled, mixed for 30 seconds to break up bacterial clumps and diluted with sterile phosphate buffered saline to an optical density of 0.5 to 0.8 read at 400 nanometers in a spectrophotometer (Coleman Jr II, Coleman Instruments, Maywood, IL) using 12 mm diameter cuvettes. Inoculum prepared in this manner contained approximately 1 X 10^9 colony-forming units of

H. somnus per ml. Calves were given 20 ml of this suspension within 30 minutes of preparing the inoculum.

EXPERIMENTAL ANIMALS: Thirty-one 1 to 5-week-old calves of mixed breeds were housed in indoor pens with concrete floors and environmental temperatures maintained between 15 and 22 C. None of the calves had complement fixing antibody titers to H. somnus, and none was shedding H. somnus in nasal secretions. All calves were challenged intratracheally with 20 ml of sterile phosphate buffered saline suspensions of H. somnus or sterile phosphate buffered saline without H. somnus (control group) via a 55 cm #5 Fr sterile polypropylene catheter (Sovereign, Monoject, Sherwood Medical, St. Louis, MO) passed through a 1 1/2" 12g needle inserted into the trachea 3 to 5 cm below the larynx. The catheter was inserted to its full depth and slowly withdrawn 6 to 8 cm as the inoculum was injected. The catheter was flushed with 50 ml of air and 20 ml of sterile phosphate buffered saline before withdrawing it from the trachea.

EXPERIMENTAL GROUPS: Thirty calves were randomly assigned to one of 6 experimental groups of 5 calves each. Groups of 5 H. somnus exposed calves and 5 control calves were killed and examined at 6 and at 72 hours post-exposure, respectively. Groups of 5 calves exposed intratracheally to H. somnus were also examined at 1 and at 24 hours

post-exposure. In addition, one control calf was examined 1 hour post-exposure. Because only 1 of 5 exposed calves had lesions at 1 hour, additional control calves were not examined at 1 hour post-exposure.

NECROPSY PROCEDURES: At necropsy, the right middle lung lobe was inflated with 2.5% glutaraldelhyde buffered with 0.1 M sodium cacodylate. The caudal segments of the right and left cranial lobes were inflated with 10% neutral buffered formalin. Both fixatives were infused at a pressure of 28 to 30 cm water. Sections of lung from other lobes were fixed in formalin by immersion. Tissues from unfixed lobes were cultured for bacteria by standard methods.

Formalin fixed lung was embedded in paraffin, sectioned at 5 micrometers and stained with haemotoxylin and eosin by standard methods. Lung for transmission electron microscopy (TEM) examination was rinsed in buffer, post fixed in 2% osmium tetroxide, dehydrated in graded steps of ethanol, cleared in acetone and embedded in Mollenhaurer's resin (Epox-Araldite mixture). [33] Sections were cut at 1 to 2 micrometers and stained with 1% toluidine blue. Selected blocks were thin sectioned at 70 to 90 nanometers, mounted on copper grids and stained with lead citrate and uranyl acetate.

Lung blocks approximately 50 mm^3 were prepared for scanning electron microscopy (SEM) examination by dehydrating through increasing concentrations of acetone and

absolute ethanol, critical point drying using liquid CO_2, mounting on stubs and sputter coating with gold to a depth of approximately 20 nm. Additional blocks were dried using hexamethyldisilazane (Sigma Chemical Company, St. Louis, MO) [34] (instead of critical point drying), mounted on stubs and sputter coated with gold or gold-paladium.

DATA ANALYSIS: To analyze the differences between bronchiolar and alveolar exudates over time, twenty randomly selected 40X microscopic fields of lung with inflammatory changes were examined and ranked according to the ratio of the types of inflammatory cells in the bronchioles and the alveoli. Sites for examination were chosen by laying a grid divided into numbered 1 mm squares on the histology slide and selecting sites using a computer generated random number list. Scores of 1 were given to alveolar and bronchiolar exudates composed primarily of neutrophils and scores of 5 were given to exudates composed primarily of macrophages. Scores of 3 were for approximately 50-50 ratios. Therefore, if all exudates in alveoli at the twenty points were primarily neutrophils, the alveolar score would be 20 (20 X 1) or if the exudates were primarily macrophages the alveolar score would be 100 (20 X 5). If no cellular exudates were present at a selected point, additional slides and points were examined until data from 20 points were collected for each calf.

Comparisons of data from different times after exposure

was done with the non-parametric Wilcoxon-Mann-Whitney Two Sample Test.[49]

Comparisons between intratracheally exposed calves and control calves were also performed at 6 hours and at 72 hours post-exposure to determine if sterile phosphate buffered saline was responsible for the lung lesion observed in H. somnus exposed calves. Twenty randomly selected lung fields were examined at 40X and ranked according to the extent and severity of fluid and cellular infiltrations. The following numerical scores were given to the lung sections at each examination site:

0 = no visible fluid or cellular exudates

1 = mild inflammation

2 = moderate inflammation; distension of alveoli and bronchioles with inflammatory cells

3 = severe inflammation; necrosis of inflammatory cells and host tissues

RESULTS

GROSS LESIONS. One of 5 H. somnus infected calves examined 1 hour post-exposure had gross lung lesions. These changes included small (up to 5 mm diameter) scattered reddened atelectatic foci in multiple lobes. The other four calves had no visible lesions.

By 6 hours post-exposure, interlobular edema was prominent in cranial ventral lobes of H. somnus infected calves with lobular patterns of greyish to reddish-grey

consolidation (Figure 3-1). The extent of the edema and consolidation varied among calves. No pleuritis was observed.

By 24 hours, the edema was diminished in severity and extent and greyish to reddish lobular atelectasis and consolidation were common in cranio-ventral locations. Occasional lobules contained foci of hemorrhage.

At 72 hours post-exposure, the gross lesions were not remarkably different than those at 24 hours. Greyish to occasionally hemorrhagic consolidation of ventral portions of cranial, middle, caudal and accessory lobes was present (Figure 3-2). The affected lung was usually sunken from surrounding parenchyma. Distinct focal areas of necrosis near the lobar bronchus were common. When these necrotic foci were near the pleural surface, focal fibrinous pleuritis accompanied the lesion. However, diffuse pleuritis was uncommon.

Tracheobronchial and mediastinal lymph nodes were grossly unaffected at 1 hour post-exposure, mildly to moderately enlarged at 6 and 24 hours and consistently edematous and enlarged at 72 hours. Congestion and hemorrhage of the respiratory lymph nodes were not observed. Tracheal and nasal mucosa was not grossly altered except for the focal wound in the trachea at the needle puncture site.

Figure 3-1. Photograph of the lung of a calf intratracheally exposed to Haemophilus somnus 6 hours previously. Interlobular edema (arrows) and early consolidation of the lung are present.

Figure 3-2. Photograph of the lung of a calf exposed 72 hours previously to Haemophilus somnus. The right cranial lobe is pneumonic and sunken from surrounding lung.

HISTOLOGIC AND ULTRASTRUCTURAL CHANGES.

ONE HOUR POST-EXPOSURE. Four of five calves examined at 1 hour post-exposure had few histologic changes. Scattered alveoli and bronchioles contained scanty cellular exudates of neutrophils and erythrocytes and amorphous debris. Alveolar walls were congested in scattered regions. Alveolar macrophages were larger and more numerous than in controls and had abundant pale basophilic cytoplasm. Bronchial epithelial cells had occasional debris on their surfaces, and bacteria could be seen in the debris or on cilia. No cilial damage to bronchial or bronchiolar epithelium was observed.

Bronchioles contained less debris but more bacteria than bronchi. Bacteria were most frequently seen on the microvilli of the central portions of non-ciliated bronchiolar epithelium or at the borders of the cilia of the ciliated bronchiolar epithelium. A few bacteria were present in alveoli and were mixed with fibrillar material. A few neutrophils were observed in bronchiolar submucosal vessels and on alveolar surfaces. There was no damage to alveolar or bronchiolar epithelium.

Bacteria on bronchiolar epithelium were separated from the microvilli and cilia by thin electronluscent spaces (Figure 3-3). The bacteria did not cause indentation or thickening of the bronchiolar epithelial plasmalemma. There were no connections between bacteria and bronchiolar epithelial cells observed and there were no attachment

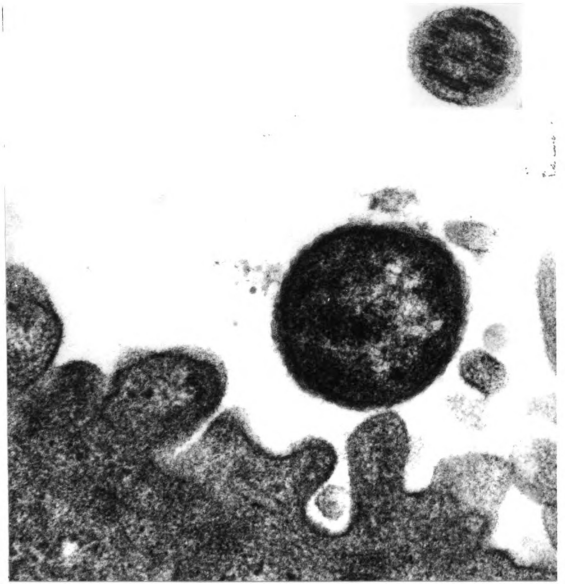

Figure 3-3. Transmission electron micrograph of a bacterium on the microvillus surface of a bronchiolar epithelial cell 1 hour after intratracheal exposure of a calf to Haemophilus somnus. No attachment organelles or alterations of the host cell membrane are visible. X 115,000.

organelles such as pili or fimbria. Patchy fibrillar material was present on the surface of bacteria, and was especially prominent in areas of bacteria to bacteria contact, or in areas of contact between microvilli and bacteria (Figure 3-4).

One calf examined at 1 hour post-exposure had numerous alveoli and bronchioles filled with edema, fibrinous fluid, neutrophils and erythrocytes (Figure 3-5). Neutrophils were numerous in bronchiolar lumens and submucosal venules. Alveolar exudation of neutrophils and lesser numbers of macrophages was generally mild with focal areas of more intense reaction. Alveolar macrophages, accompanying the fibrinous and hemorrhagic alveolar exudation, were enlarged 2 to 3 times normal size with pale basophilic granulated and vacuolated cytoplasm. These macrophages contained fibrinous and erythrocytic debris and bacteria. Neutrophils on alveolar and bronchiolar surfaces were also filled with bacteria, erythrocytes and fibrin. Numerous neutrophils were also present in alveolar walls and in alveolar lumens. Bacteria were found more easily on bronchiolar surfaces in this calf than in other calves examined at 1 hour.

Since only one calf had lesions at 1 hour post-exposure, lung scores were not tabulated for this group.

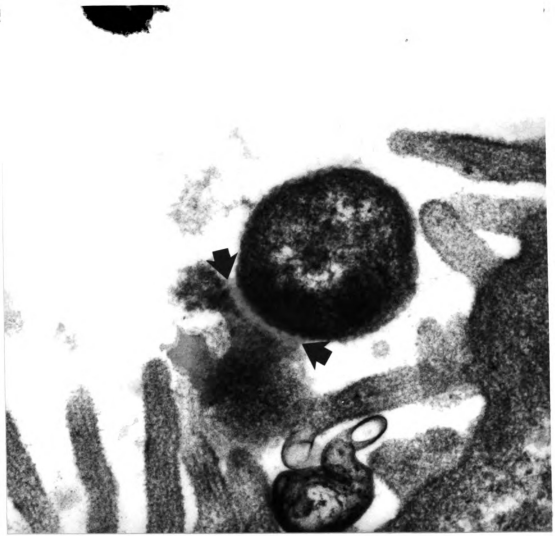

Figure 3-4. Transmission electron micrograph of a bacterium on the surface of bronchiolar epithelium 1 hour after exposure of a calf to Haemophilus somnus. Extracellular material is present at the arrows between the bacterium and surrounding structures. X 115,000.

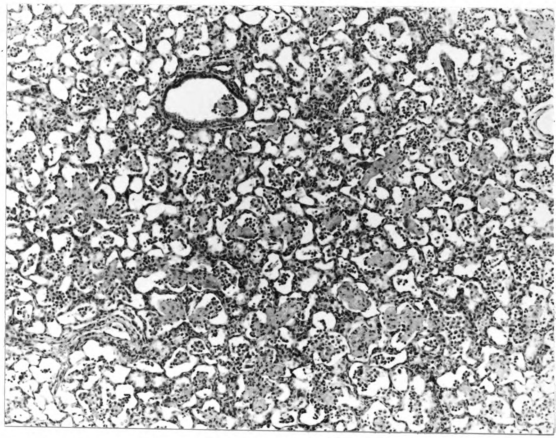

Figure 3-5. Photomicrograph of the lung of a calf 1 hour after exposure to Haemophilus somnus. Erythrocytes, fibrin, a few neutrophils and edema fluid are present in alveoli. HE. X 295.

SIX HOURS POST-EXPOSURE. By 6 hours, the histologic changes in all five calves were extensive and varied only in the severity of the cellular exudation. Bronchioles were consistently filled with neutrophils (mean bronchiolar score = 22.2 $\pm$1.64) fibrin and a few scattered erythrocytes. Numerous neutrophils were also present in bronchiolar submucosal blood vessels and in the bronchiolar mucosa. In many lobules, there were only a few neutrophils in alveoli while there were many neutrophils in the bronchioles. In other lobules, both bronchioles and alveoli were filled with neutrophils (mean alveolar score = 27.6 $\pm$1.82). Neither alveolar nor bronchiolar epithelium was necrotic. Perivascular and interlobular lymphatics were markedly dilated with proteinaceous fluid and occasionally contained low numbers of neutrophils. Blood vessel walls (arteries, arterioles, veins and venules) often contained neutrophils especially in regions where alveoli were severely inflamed.

Most affected bronchi contained only a few neutrophils and little fibrin. A few neutrophils were also present in bronchial mucosa or in submucosal vessels. The pleura was unaltered except for mild dilatation of subpleural lymphatics with pink staining fluid. Tracheobronchial lymph nodes contained massive numbers of neutrophils in subcapsular, cortical and medullary sinusoids.

The differences in the inflammatory respone between alveoli and bronchioles was readily apparent in SEM

specimens (Figure 3-6) from most lobules. Phagocytic cells covered bronchiolar surfaces and cilia and microvilli were often bent and laying on the surface rather than projecting outward (Figure 3-7). Bacteria were not readily visible on SEM examination, but they could occasionally be found adjacent to neutrophils.

Close interdigitation of bronchiolar epithelial projections and surface neutrophils was observed in TEM sections (Figure 3-8). Few bacteria could be identified on bronchiolar surfaces but moderate numbers of bacteria and degenerating bacteria were within the neutrophils on bronchiolar and alveolar surfaces. Occasionally cilia were also identified in neutrophil phagosomes. Bacteria were most easily identified in bronchioles and alveoli in regions of intense inflammation. Numerous neutrophils were present between bronchiolar (Figure 3-9) epithelial cells apparently migrating into the lumen. Alveolar walls were thickened with dilated capillaries and edema, and neutrophils were present in interstitial spaces. Neutrophils in the alveolar lumen were only occasionally in contact with alveolar surfaces.

TWENTY FOUR HOURS POST-EXPOSURE. By 24 hours, the edema in interlobular and subpleural lymphatics contained numerous fibrin strands and thrombi which enmeshed moderate to large numbers of neutrophils and erythrocytes. Alveoli in more intensely inflamed regions contained numerous

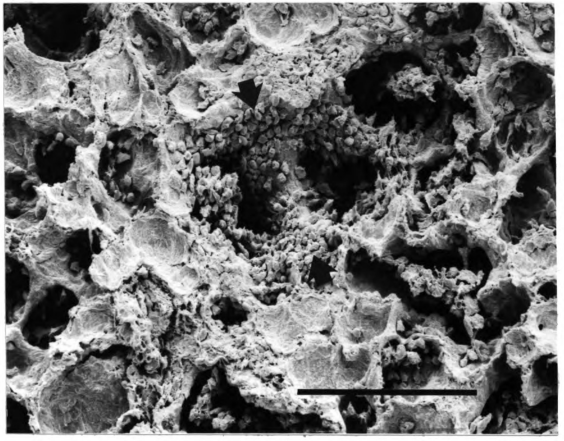

Figure 3-6. Scanning electron micrograph of calf lung 6 hours after exposure to Haemophilus somnus. Numerous neutrophils line the surfaces of the bronchioles (arrows) while few inflammatory cells are present in the alveoli. Bar = 100 micrometers.

Figure 3-7. Scanning electron micrograph of the bronchiolar surface of a calf, 6 hours after intratracheal exposure to Haemophilus somnus. The cilia are bent and lying on bronchiolar epithelial surfaces while neutrophils are in close contact with the bronchiolar epithelium. Bar = 20 micrometers.

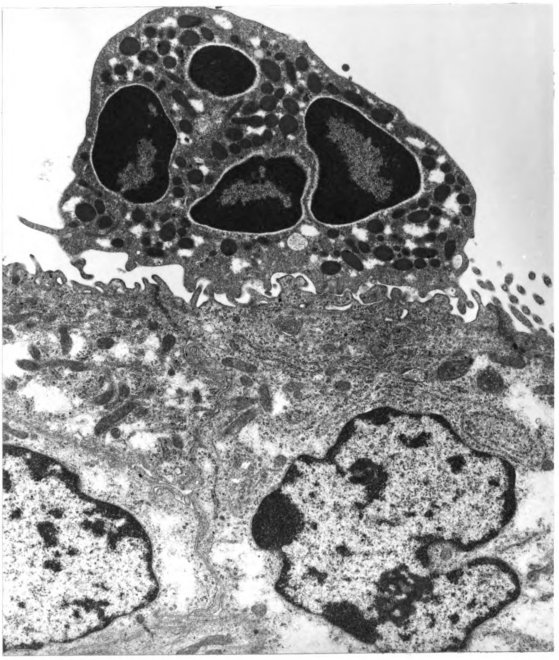

Figure 3-8. Transmission electron micrograph of close interdigitation of a neutrophil with the microvillus surface of a bronchiolar epithelial cell 6 hours after intratracheal exposure of a calf to Haemophilus somnus. X 16,000.

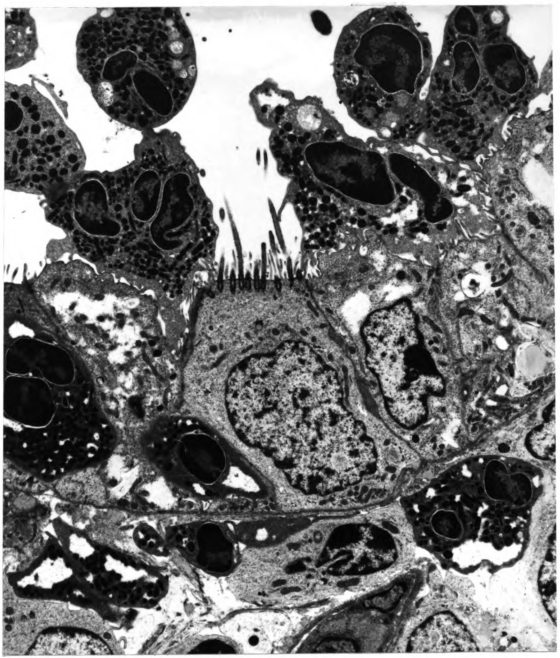

Figure 3-9. Transmission electron micrograph of the bronchiolar mucosa of a calf exposed to Haemophilus somnus 6 hours previously. Numerous neutrophils are present on the bronchiolar surface, between epithelial cells and in the submucosal interstitium. X 6,700.

rounded and deeply basophilic cells with indistinct nuclear detail. These changes were characteristic of dying cells. Alveolar fluid was more deeply eosinophilic in these regions of exudate necrosis and the alveolar walls were markedly congested.

In less intensely inflamed lobules, bronchioles contained neutrophilic exudates but surrounding alveoli were not inflamed. In moderately affected regions, bronchioles and alveoli were filled with inflammatory cells but there was no necrosis. There were increased numbers of macrophages in alveolar exudates (mean alveolar score = 51.6 $\pm$12.03) but fewer in bronchiolar exudates (mean bronchiolar score = 32.2 $\pm$1.48). Inflammatory cells were in low numbers in the adventitia of arteries in intensely affected regions. Scattered bronchi contained scanty neutrophilic exudates on surfaces and a few epithelial and submucosal inflammatory cells similar to changes observed at 6 hours.

SEVENTY TWO HOURS POST-EXPOSURE. By 72 hours, the histologic changes formed 1 of 3 fairly distinct patterns classified as mild, moderate or severe.

Mild changes were characterized by a slight increase in the numbers of neutrophils and macrophages in alveoli and bronchioles with no dilatation of interlobular septal lymphatics.

Moderate changes were characterized by a mixture of neutrophils and macrophages filling and occasionally

distending bronchioles and alveoli. Inflammatory cells were abundant in bronchiolar mucosa and submucosal vasculature. Interlobular septa were mildly distended with fibrinocellular fluid or were not affected. Necrosis of exudates and host tissues was not apparent. In moderately affected lung, loss of bronchiolar epithelial cilia and microvilli was frequently associated with the presence of cellular exudates (Figure 3-10). Bronchiolar epithelium was generally intact. Bacteria were rarely found outside phagocytic cells, and only a few were found within phagocytes.

Severe changes were characterized by the filling of bronchioles and surrounding alveoli with mixed inflammatory cells, fibrin and erythrocytes. Necrosis of inflammatory cells and host tissues was common in bronchioles and in alveoli surrounding these severly affected bronchioles. Marked dilatation of interlobular and subpleural lymphatics with fibrinocellular thrombi surrounded severely affected regions. Vascular inflammation, thrombosis and necrosis were common in these areas. In severly affected regions, loss of bronchiolar epithelium was frequent (Figure 3-11). This was always associated with the presence of high numbers of inflammatory cells, many of which were necrotic. Alveolar basement membranes were denuded with fibrillar material covering the surfaces. Inflammatory cells in both bronchioles and alveoli contained numerous bacteria, cellular debris and fibrin.

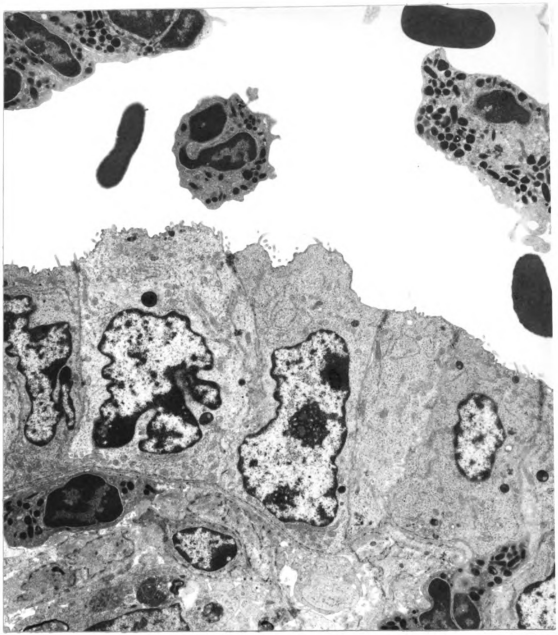

Figure 3-10. Transmission electron micrograph of the bronchiolar epithelium 72 hours after intratracheal exposure of a calf to Haemophilus somnus. Few cilia and microvilli are visible, but the epithelium is intact. Neutrophils and erythrocytes are present in the bronchiolar lumen. X 6,500.

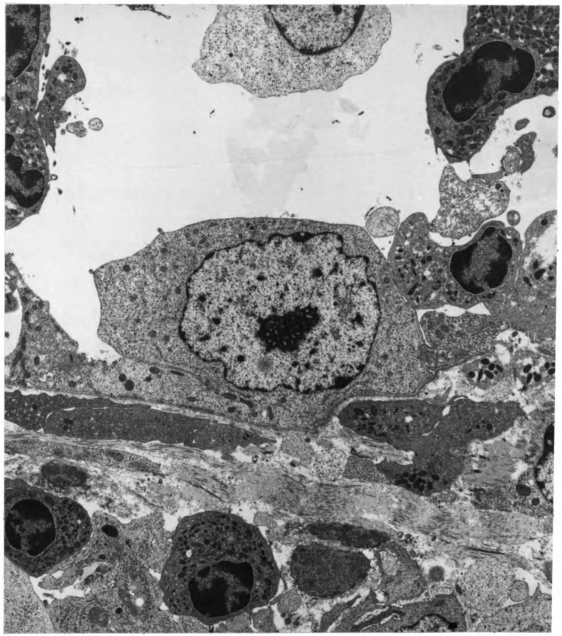

Figure 3-11. Transmission electron micrograph of the bronchiolar mucosa in a severly affected region of a lung of a calf exposed to Haemophilus somnus 72 hours previously. The bronchiolar epithelium remaining is rounded and lacks surface projections. X 6,800.

In the lungs from calves examined at 72 hours, macrophages predominated in the alveolar reactions (mean alveolar score = 68.8 ±6.06) while in bronchioles, neutrophils still predominated (mean bronchiolar score = 30.0 ±5.70).

No significant changes were seen in the lungs of control calves.

Haemophilus somnus was isolated from the lungs of all calves except for one calf examined at 24 hours and the 11 non-exposed controls.

COMPARISON OF ALVEOLAR AND BRONCHIOLAR SCORES. Alveolar scores were not sigificantly different from bronchiolar scores at 6 hours but were significantly higher ($p = 0.01$) than bronchiolar scores at 24 and 72 hours. When all three time groups (6, 24 and 72 hours) were combined and bronchiolar scores compared to alveolar scores, bronchiolar scores were significantly lower ($p = 0.01$). The difference between alveolar scores and bronchiolar scores was least at 6 hours and greatest at 72 hours. Bronchiolar scores at 24 and 72 hours were significantly higher ($p = 0.01$) than bronchiolar scores at 6 hours but scores at 24 and 72 hours did not differ significantly from each other.

Alveolar scores at 24 hours were significantly higher than 6 hour scores and 72 hour scores were significantly higher ($p = 0.01$) than 6 hour and 24 hour alveolar scores.

These data support the histologic observations that

macrophages increased relative to neutrophils over time in the bronchiolar and alveolar exudates and that the alveolar exudates contained more macrophages at 24 and 72 hours than did bronchiolar exudates.

EXTENT AND SEVERITY SCORES. Extent and severity lung scores were used to test the null hypotheses that the observed inflammatory changes were due to the sterile phosphate buffered saline without H. somnus bacteria and that the extent and severity of the lesions was related to the time of examination after exposure.

Extent and severity scores for H. somnus exposed calves at 6 hours post-exposure ranged from 21 to 41 (28.4 $\pm$8.02) and for 72 hour calves from 28 to 41 (33.6 $\pm$5.32). There was no significant difference between these two groups.

Extent and severity scores for control calves ranged from 0 to 2 and there was no significant difference between 6 hour and 72 hour controls. There were, however, significant differences ($p = 0.01$) between H. somnus exposed calves and controls at both 6 and 72 hours post-exposure.

As seen ultrastructurally, the earliest response of the bovine lung to intratracheally administered sterile phosphate buffered saline containing H. somnus bacteria was the cytoplasmic enlargement of alveolar macrophages with ingested bacteria. This was quickly followed by multifocal congestion of alveolar capillaries accompanied by fluid and erythrocytes leaking into alveoli. Large numbers of

neutrophils migrated into alveoli by 1 to 6 hours accompanied by more extensive bronchiolar neutrophilic exudation. Neutrophils appeared to be migrating from both the post-capillary venules of the bronchiolar submucosa and from the alveolar capillaries. Bronchial contribution to these inflammatory exudates was apparently minimal.

By six hours post-exposure, proteinaceous fluid filled many alveoli accompanied by many neutrophils. Septal and pleural lymphatics were dilated with edema fluid containing little visible fibrin. Bronchiolar filling with neutrophils was prominent even in lobules without alveolar exudation.

By 24 hours, septal and subpleural lymphatics contained fibrin clots, erythrocytes and neutrophils. In intensely infiltrated alveoli, phagocytic cells were becoming necrotic, and deeply eosinophilic fluid in surrounding alveoli was prominent. Macrophages were present in increasing numbers in alveolar exudates especially in moderately affected areas. Neutrophils predominated in bronchioles, and in many lobules there was neutrophilic bronchiolitis without alveolar exudation.

By 72 hours, the macrophage was the predominant inflammatory cell in alveoli while the neutrophil predominated in the bronchioles. Inflammatory vasculitis was present in severly affected areas.

DISCUSSION

The most striking finding of this study was that the cellular composition of bronchiolar and alveolar exudates differed significantly 6, 24 and 72 hours after H. somnus exposure and that the differences became greater as time increased. Cellular exudation from these two sites probably does not reflect the same pathogenetic mechanism. The apparent source of the neutrophils and macrophages was the post-capillary venules in the bronchioles and the capillaries in the alveoli, respectively. The bronchiolar epithelial cells and the submucosal venules in cattle are probably more permeable to cellular migration and fluid than either the alveolar epithelial or capillary endothelial cells, thus producing differences in the inflammatory responses between alveoli and bronchioles.

In most tissues cellular and fluid exudation in acute inflammation is from post-capillary venules which have less "tight" cell to cell junctions than capillaries. [56] The lung appears to be different in this regard. [56] Neutrophils migrate across lung arterioles and capillaries as well as venules. [28,56] Alveolar capillary blood is derived almost entirely from the pulmonary artery while blood in the submucosal venules of the bronchioles and bronchi is from both the pulmonary and bronchial arteries. [38] Alveolar capillaries open into post-capillary venules in pleated alveolar corners [18] and in ruminants, swine and horses, the venules empty into pulmonary veins accompanying the

bronchial tree. [26] Post capillary venules in the terminal airways of cattle receive blood from both the bronchial and pulmonary circulation, [31,38] and bronchial capillaries anastomose with pulmonary capillaries at the level of the terminal bronchioles. [38]

Capillary endothelial cells in the bronchiolar submucosa form tight junctions, and capillaries are relatively few in number. [38] Most of the microvasculature in the bronchiolar submucosa is composed of venules with pericytes and smooth muscle surrounding the endothelium. [38] The venular cell to cell junctions are relatively loose but are enclosed by a continuous basement membrane. [38] Vasoactive amines (i.e. histamine), bradykinin, and lipopolysaccharide endotoxin cause bronchiolar submucosal venules to become highly permeable to tracers. [38] This effect is absent in the pulmonary microvasculature. [38] The venular end of the pulmonary capillary bed has the most permeable of the 3 types of vascular junctions present in the alveolar capillaries. [43] The arteriolar segment of this bed is the least permeable. [43]

In contrast, the alveolar epithelium is impermeable to all tracers and its junctions are composed of continuous complex networks of junctional fibrils [43] which disaggregate when exposed to proteolytic enzymes. [43] Airway epithelia lining bronchioles have less tight junctions than the ciliated epithelium lining the trachea and bronchi. [42]

It therefore appears that both the bronchiolar

vasculature (venules) and the bronchiolar epithelium are more permeable to tracers and probably to inflamatory cells than either the alveolar capillary endothelium or the alveolar epithelium. This likely produces the differences in fluid and cellular exudation from these sites observed in these experiments. Alveolar fluid and cellular exudation occurred as early as 1 to 6 hours post-exposure in our calves but was not as widespread throughout lung tissue as was the cellular exudation into bronchioles. Bronchial exudation was minimal at all stages. The pattern of exudation seen in these calves suggests that when the lung is severly injured, both alveolar and bronchiolar sites become sources of neutrophils. In severe injury the alveolar exudation may dominate the histologic lesions. When injury is less severe, bronchiolar venules may allow neutrophils to pass while alveolar capillaries may not, and the histologic lesion becomes a neutrophilic bronchiolitis with little alveolar response.

In rabbits given carbon particles intratracheally (a relatively mild injury), neutrophils appeared in the bronchioles several hours before appearing in the alveoli. [7] This further supports the hypothesis that bronchiolar and alveolar neutrophils originate from two different sites. Since alveolar contents are cleared via the tracheobronchial tree by the mucociliary apparatus, [24] bronchiolar exudates may also contain cells of alveolar origin.

An alternative explanation of the differences between

bronchiolar and alveolar neutrophil exudation is the possible selective localization of H. somnus bacteria, H. somnus chemotaxins or host generated chemotaxins in the bronchioles. In this study, H. somnus could be identified more easily on bronchiolar surfaces than in alveoli in calves examined 1 hour post-exposure, but bacteria were in both locations in calves examined at later times. It seems unlikely that the continued presence of H. somnus in bronchioles is the major reason for the persistent neutrophilic bronchiolitis. Haemophilus somnus was observed in both bronchioles and alveoli where epithelial cells were poorly ciliated or non-ciliated. The bacteria appeared to be deposited randomly and did not attach to bronchiolar epithelium by specialized organelles such as pili or fimbria as has been reported for many other bacteria. [34,53] Epithelial surfaces in the vicinity of deposited organisms did not develop indentations of cell surfaces, or thickenings of the plasma membrane, and did not appear to be morphologically altered by bacteria 1 hour post-exposure. [34] The attachment and localization of bacteria without specialized attachment organelles to epithelial cell surfaces may be aided by surface active components such as extracellular polysaccharides (glycocalyx). [11]

In order for bacteria to colonize a mucosal surface, the organism must progress sequentially through several steps. [16] It must 1) make contact with the surface, 2) penetrate the surface material either passively or

actively, 3) adhere to the mucosal surface and 4) multiply on or penetrate through the mucosal epithelium. [16]

In the respiratory tract, contact of aerosolized bacteria with the surface is aided by deposition mechanisms such as impaction, sedimentation and diffusion. [13] Particles the size of H. somnus are largely deposited in alveoli [13] or at the bifurcations of the terminal airways. [8] The role of deposition mechanisms following administration of bacteria suspended in fluid has not been determined.

Penetration of the organism through lung surface material differs with the lung site. In lungs of ruminants, mucus production is limited to the trachea, the bronchi and the larger bronchioles while terminal airways are lined with non-mucus secreting cells. [29,30,39,40] Although non-ciliated bronchiolar epithelium (Clara cell) of many species produces carbohydrate material [29] which contributes to the hypophase or sol layer of the surface mucus [23] of larger airways, the non-ciliated bronchiolar epithelium of sheep and cattle produce little surface material. [29,40] This non-ciliated bronchiolar epithelial cell in cattle resembles the fetal Clara cell of many other species. [39] The surfaces of terminal airways of cattle are probably lined with surfactant produced by alveolar epithelium.

The gel layer of mucus is impermeable to water, [13] and surfactant also repels water. [32] Therefore the saline used to suspend the H. somnus inoculum in these experiments would not be expected to significantly alter airway surface

materials allowing artificial bacterial attachment. The thinner surfactant layer is more permeable than the mucus layer, which may explain the more frequent presence of H. somnus on bronchiolar and alveolar surfaces than on bronchial surfaces at 1 hour post-exposure in these experimental calves.

The third step in colonizing a mucosal surface is adherence. [16] The outer membrane of bacteria is generally highly charged and is repelled by electrostatic forces from cell surfaces. [5] The extracellular glycocalyx of bacteria forms a hydrophilic extension of the charged surface, allowing the repulsive electrostatic forces to be overcome and cell to cell association to occur. [5,41] In this study, electron dense material resembling glycocalyx [11] was present between adjacent bacteria and between bacteria and bronchiolar cell surfaces. Polysaccharides, polyribose and ribonucleotides have been extracted from intact H. somnus organisms, [10,37] presumably from the cell surface. These may be components of an extracellular matrix important in the attachment of H. somnus to cell surfaces. Haemophilus somnus glycocalyx has not been demonstrated by others. [50, 51,55]. Further investigation of the possible presence of a glycocalyx is needed.

The fourth step in mucosal colonization by a bacteria is the multiplication of the organism on the surface or the penetration of the bacteria into the epithelium. H. somnus may be present in pneumonic lungs in numbers as high as

10^8 per cubic centimeter of pneumonic lung [19] suggesting that multiplication in the lung occurred. Penetration of the epithelium by H. somnus was not observed in this study, but adherence to arterial endothelium and inclusion of the bacteria in superficial vacuoles of the endothelium [51] suggest that the bacteria either penetrated endothelium or were phagocytized. At times after 1 hour post-exposure, changes in epithelial cells may have been induced by the inflammatory response [44] or by the bacteria. [22]

Bacterial-derived chemotaxins from H. somnus have not been identified but their possible existence has not been disproven. Although most bacteria produce formylated oligopeptides which stimulate the neutrophils and macrophages of most species to migrate unidirectionally, [36] bovine neutrophils do not respond to formylated oligopeptides [15, 20] but do respond to other products of Gram-negative bacteria. [15] Until H. somnus chemotaxins are identified it is impossible to determine selective localization of chemotaxins in bronchiolar locations.

Host derived neutrophil chemotaxins include macrophage derived factors, [47] leukotrienes (especially leukotriene B4), [36] and activated complement. [36,56] It is unlikely that macrophage derived factors localize selectively in bronchioles since alveolar macrophages are normally more numerous in alveoli. Also in this study, macrophages were more numerous in alveoli than bronchioles in diseased lung. The selective localization of activated complement in lung

sites has not been investigated.

Neutrophils are an abundant source of arachidonic acid metabolites including the leukotrienes. [36] Neutrophil-derived leukotrienes may potentiate and continue a neutrophilic reaction, but this does not explain the observed differences between bronchiolar and alveolar responses in these calves since both reactions began as neutrophil dominated reactions. Potent oxidants cause increased arachidonic acid metabolism in airway epithelium. [35] Arachidonic acid is readily converted to lipoxygenase products such as leukotriene B4 resulting in powerful chemotactic activity in the airway epithelium. [35] This may be a means of continued neutrophil chemotaxis into airways while in alveoli lacking epithelial-produced leukotrienes, the cellular exudates become more monocytic. Oxidant injury of the airways could occur from superoxide anion, singlet oxygen and other reactive oxygen species produced by neutrophils. [21,47]

Activated complement fragments (especially C5a, C5a des Arg, and C3b) are potent neutrophil chemotaxins. [21] Instillation of activated complement into the lungs of rabbits produced acute hemorrhagic alveolitis with neutrophil accumulation, fibrin deposition and edema. [21] Bronchiolar lesions were not described. This suggests that complement has more effect on alveolar than bronchiolar exudation. The resolution of most of the edema fluid by 24 hours in these calves followed the pattern described for

complement induced lung injury, [21] however, the continuing cellular exudation suggests that more than just complement is involved in H. somnus injury to pulmonary tissues.

Bacterial factors such as endotoxin may activate complement. [25] Endotoxin of P. hemolytica or Eschericia coli instilled into sheep lungs produced acute hemorrhagic pneumonia which progressed to neutrophilic infiltration by 24 hours. [9]

Although others have reported that bovine neutrophils phagocytize but do not kill H. somnus in-vitro, [12] evidence of both phagocytosis and intracellular digestion of H. somnus as early as 1 hour by macrophages and at 6 hours by neutrophils was observed in these calves. It was difficult, however, to quantitate this response. It may be that factors needed by phagocytes for intracellular killing of H. somnus were not present in in-vitro systems.

Areas of the most intense bronchiolar and alveolar fluid and cellular responses in early lesions probably correspond to the regions of necrosis and vasculitis seen in later lesions. In these areas, bacterial organisms could be easily found on light microscopic and transmission electron microscopic examinations. If cytotoxins are elaborated by H. somnus as has been suggested, [27] the amount of cytotoxin released is probably related to the number of organisms and their growth phase. [3,6] Liggett et al [27] demonstrated an in-vitro relationship between the number of H. somnus cells and alveolar macrophages with

cytotoxicity (macrophage death) which occurred at a 10:1 ratio of bacteria to alveolar macrophages. Liggett *et al* also observed that it was necessary for the bacteria to be ingested by alveolar macrophages before cytotoxicity occurred. The supernatant of *H. somnus* growth did not have significant effects on alveolar macrophages.[27] Further details have not been published. Cyprynski[12] did not report cytotoxic effects in bovine neutrophils following *H. somnus* ingestion but used *H. somnus* cells and phagocytes in equal numbers.

The occurrence of necrosis and vasculitis in bovine lungs following exposure to *H. somnus* is probably related to the numbers of bacteria at the necrotic site and the relative production and potency of their cytotoxin. Since details of *H. somnus* alveolar macrophage cytotoxicity studies have not been published, it is difficult to compare the relative potency of *P. hemolytica* and *H. somnus* cytotoxins. *Pasteurella hemolytica* in log phase growth produces a potent cytotoxin which is present in culture supernatants and is capable of killing both alveolar macrophages[54] and neutrophils at 10:1 ratios of bacteria to cells.[3,6] This differs from most other bacterial leukotoxins which are cell associated and released only after cell death.[45] Severe necrosis and formation of "streaming" patterns of pulmonary inflammatory cells as early as 6 hours after exposure to *P. hemolytica* have been reported.[48] *Pasteurella hemolytica* cytotoxin resulted in

death and cytolysis of bovine neutrophils after only 20 minutes exposure in-vitro. [3,6]

Conversely, living H. somnus cells must be ingested by phagocytes before cytotoxicity occurs, [27] and supernatants are not toxic. It therefore appears that a major difference between P. hemolytica and H. somnus is the presence of a potent extracellular neutrophil and macrophage cytotoxin produced by P. hemolytica while H. somnus produces cytotoxicity only after phagocytic ingestion.

In this study, neutrophils attached to bronchiolar cell surfaces, developing close and intimate contact with epithelial projections such as cilia and microvilli. Indentations of neutrophil cytoplasmic membranes containing epithelial cell surface projections were common. Neutrophil products (superoxide anion, proteases, peroxidases, etc.) may escape into the immediate environment of the epithelial cell projections when phagosome-lysosome fusion occurs with incompletely closed phagosomes. [14,47] Dying and degenerating neutrophils may also release proteases and other products into the immediate environment. [47] The observation in this study that cilia and microvilli were reduced or absent in bronchioles with neutrophilic exudates at 72 hours suggests that persistent inflammatory exudates have detrimental effects on cell surface projections. Detachment of epithelial cells was most prominent in regions of neutrophils in the bronchiolar mucosa and in the lumens. Many detaching cells did not appear to be irreversibly

damaged and had only minimal cytoplasmic and nuclear changes. Proteases, especially elastase, from neutrophils are important mediators of epithelial cell detachment. [21] Detachment of epithelium and degenerative changes in epithelium were present as early as 6 hours but became much more prominent at 72 hours and were most evident in severly affected lobules with the most intense inflammatory cell infiltrates.

In conclusion, H. somnus produced pneumonia which followed a pattern of acute fluid exudation, followed quickly by neutrophil influx into bronchioles and alveoli. Neutrophil infiltration into bronchioles without significant alveolar inflammation was the major change observed in less severly affected regions. Over time, the alveolar exudates became principally composed of macrophages while bronchiolar exudates remained primarily neutrophilic. Necrosis of exudates and host tissues began by 24 hours and was extensive by 72 hours in regions of relatively high bacterial numbers. Vasculitis was observed only in areas of necrosis.

The neutrophilic bronchiolitis which occurs with H. somnus pneumonia probably relates more to the mechanisms controlling the inflammatory response following mild or limited injury in bronchioles versus alveoli, rather than to the persistence of bacteria or bacterial products in those regions. It is also likely that bacterial toxins acting over time contribute to more severe lung injury.

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CHAPTER FOUR

COMPARISON OF THE *IN-VITRO* ATTACHMENT OF *HAEMOPHILUS SOMNUS* TO BOVINE BRONCHIOLAR AND ALVEOLAR EPITHELIUM.

ABSTRACT

Neutrophilic bronchiolitis is a characteristic feature of natural and experimental Haemophilus somnus pneumonia. One possible mechanism for the development of this bronchiolitis is the selective attachment and colonization of H. somnus to bronchiolar epithelial surfaces in preference to alveolar sites. We tested this hypothesis by examining, with a scanning electron microscope, lung maintained in an in-vitro explant system and inoculated with viable H. somnus. Haemophilus somnus inoculated into bovine lung explants, maintained in minimal essential medium, colonized the alveolar surfaces after 4 hours in significantly higher numbers ($p = 0.01$) than the bronchiolar surfaces. Bronchiolar epithelium remained unaltered for up to 6 hours while alveolar epithelium colonized with H. somnus detached and became necrotic. From these data we conclude that the usual lesions in naturally occurring H. somnus pneumonia are not the result of selective localization or persistence of the bacteria within bronchioles. These findings also indicate that the presence of H. somnus in alveoli, at least in this artificial environment, results in alveolar epithelial damage, suggesting a role for H. somnus products in alveolar, but not bronchiolar injury.

INTRODUCTION

Haemophilus somnus pneumonia is characterized by neutrophilic bronchiolitis often accompanied by alveolar exudates containing primarily macrophages in both the experimental and naturally occurring disease (Andrews, JJ: thesis, chapters 2 & 3). [2,3,12,11] In experimental infections, the alveolar exudates became more monocytic over time, while bronchiolar exudates contained mainly neutrophils (Andrews, JJ: thesis, chapter 3).

Several possible explanations exist for this difference between bronchiolar and alveolar responses. One hypothesis is that H. somnus selectively localizes on bronchiolar epithelium in preference to bronchial or alveolar surfaces and subsequent inflammatory reaction centers on bronchioles. Adherence of H. somnus to endothelial and epithelial surfaces has been associated with virulence. [30,31] Virulent strains of H. somnus were more adherent in-vitro to bovine turbinate epithelium than were avirulent strains. [31] Haemophilus somnus was also more adherent in-vitro to arterial endothelium than were Eschericia coli or Salmonella typhimurium. [30] Adherent H. somnus also produced degenerative endothelial changes which exposed underlying basement membrane. [30] One hour after experimental intratracheal exposure of calves, H. somnus could be found most frequently on bronchiolar surfaces (Andrews, JJ: thesis, chapter 3).

Experiments which compare the colonization of various

anatomical sites by H. somnus in lung tissue might help explain the predisposition for bronchiolitis in H. somnus infected lungs. This paper reports that significant colonization of alveoli, in preference to bronchioles, occurs in bovine lung explants up to 6 hours after inoculation. Degenerative changes are present in alveolar epithelium but not in bronchiolar epithelium.

METHODS AND MATERIALS

BACTERIAL INOCULUM. Haemophilus somnus organisms (strain ISU 156-83) were washed with sterile phosphate buffered saline from brain heart infusion agar (supplemented with 10% bovine blood and 0.5% yeast extract) after 18 hours growth at 37 C in humidified incubators containing atmospheres supplemented with 10% CO_2. The optical density of these suspensions was adjusted to 0.5 in 12 mm diameter cuvettes read at 400 nanometers in a spectrophotometer (Coleman Jr. II, Coleman Instruments, Maywood, IL) and diluted 1:10 to produce a suspension containing approximately 1 X 10^8 colony-forming units H. somnus/ml. This suspension was used to inoculate tissue culture wells containing lung explants.

TISSUE CULTURE MEDIUM. Each well of the tissue culture plates contained 8 ml of MEM (GIBCO Laboratories, Madison, WI). The MEM was prepared with 8.8 gm. sodium bicarbonate, 38.44 gm. MEM powder (GIBCO), 40 ml sodium pyruvate

solution (GIBCO), 40 ml lactoalbumin hydrosolate (GIBCO), 24 ml L-glutamine (GIBCO) and sterile water sufficient to make 4 L of solution. Five to ten ml of 1N hydrochloric acid was added as necessary to adjust the pH to 7.1. The medium was prewarmed in the tissue culture plates to 37 C for 15 to 30 minutes prior to inoculation.

COLLECTION OF LUNG TISSUE. Four 2 to 5-day-old dairy calves with no visible lung lesions were used as sources of lung tissue. None had circulating complement fixing antibodies to H. somnus. After euthanasia the thoracic cavity was opened aseptically as quickly as possible and the right middle lung lobe severed at its base with sterile scissors. This lobe was immediately inflated with 70 ml MEM via the lobar bronchus. The bronchus was ligated and the entire lung lobe placed in a sterile plastic bag and transferred to a laminar flow hood containing prepared tissue culture plates. One slice of lung approximately 5 mm square and 3 mm thick was placed in each well of four six-well tissue culture dishes (Costar Tissue Culture Cluster 6, Costar, Cambridge, MA) containing 8 ml of MEM.

HARVESTING AND PROCESSING LUNG EXPLANTS. After the specified incubation time, the culture medium from each well was removed and cultured for bacterial growth. The medium was replaced with cold 2.5% glutaraldehyde buffered with 0.1M sodium cacodylate and the lung fixed for 2 hours. Blocks

of lung were then rinsed twice in buffer, and dehydrated through increasing concentrations of acetone. The solution was then changed to absolute ethanol in 4 steps. The blocks were critical point dried or dried with hexamethyldisilazane (Sigma Chemical Company, St. Louis, MO), mounted on stubs, sputter coated with gold or gold paladium to a depth of approximately 20 nm and examined with a scanning electron microscope (Stereoscan 200, Cambridge Instruments, Cambridge, England).

EXPERIMENTAL DESIGN. A single block of lung was placed in each tissue culture well. Three of the six wells were inoculated with 0.1 ml of phosphate buffered saline containing approximately 1 X 10^8 H. somnus/ml. The other three wells in each plate were controls and were inoculated with 0.1 ml sterile phosphate buffered saline with no H. somnus organisms. The culture plates were placed on an orbital shaker (TekTator V, Tekpro, American Hospital Supply Corp., Evanston, IL) set at 30 rotations per minute in a humidified incubator (37 C and 5% CO_2). One of the 4 tissue culture plates was removed from the incubator at 1, 2, 4 and 6 hours and the tissues processed for microscopy. This entire lung explant process was performed on 4 separate occasions. For each time of harvest, 12 blocks of lung inoculated with H. somnus and 12 control blocks were available for comparison.

In addition, four sets of lung explants in tissue

culture plates were prepared as above except that a higher number of H. somnus (1×10^9) were inoculated into half the wells, and the other 12 wells served as controls. All these were harvested after one hour of incubation.

COLLECTION AND ANALYSIS OF DATA. Five different alveolar and bronchiolar regions of four lung blocks were selected for bacterial counts at each time period. The regions for counts were selected on the basis of their visibility with the SEM. The contour of lung structures made counting bacteria difficult unless the surface to be examined was roughly parallel to and near the surface of the block.

The average number of attached bacteria in five different 20 micrometer square areas was determined for bronchioles and alveoli in each block. The number of bacteria on bronchiolar surfaces was compared to the number of bacteria on alveolar surfaces at each time period using a Split Plot ANOVA design with time and location as treatments or factors. [29]

RESULTS

Lung incubated in MEM retained its ultrastructural characteristics for up to 6 hours with minimal changes. Bronchiolar epithelium remained attached with only occasional necrotic cells observed at 6 hours. Surfaces of non-ciliated bronchiolar cells were more clumped at 4 to 6 hours than in earlier samples, and erythrocytes were often

present on airway surfaces. Alveolar epithelium was more frequently altered than bronchiolar epithelium in control blocks. Gapping of cell to cell junctions and detachment of type I pneumocytes were occasionally observed in 4 and 6 hours samples and were more frequent in critical point dried specimens than in samples dried with hexamethyldisilazane. The majority of the bronchiolar and alveolar epithelium remained intact even in the presence of bacterial contaminants (Micrococcus spp.) as late as 6 hours (Figure 4-1).

In lung explants inoculated with live H. somnus cells, only low numbers of bacterial organisms were present on bronchiolar surfaces at any time of harvest (Figure 4-2). No evidence of damage to cilia, microvilli or cell junctions was observed (Figures 4-2 and 4-3). The relative lack of H. somnus cells on bronchiolar surfaces was in striking contrast to abundant H. somnus in alveoli at 4 and 6 hours post-inoculation (Figure 4-4). The number of bacteria on alveoli versus bronchioles was significantly greater ($p = 0.01$) at 4 and 6 hours (Table 4-1). After 2 hours incubation, bacterial numbers on both alveolar and bronchiolar surfaces increased significantly with time.

By one hour following inoculation with H. somnus, alveolar epithelium was undergoing degenerative changes and by 2 hours was detaching from basement membranes even though bacterial numbers were not high. By 4 and 6 hours numerous H. somnus were present in alveoli and there was

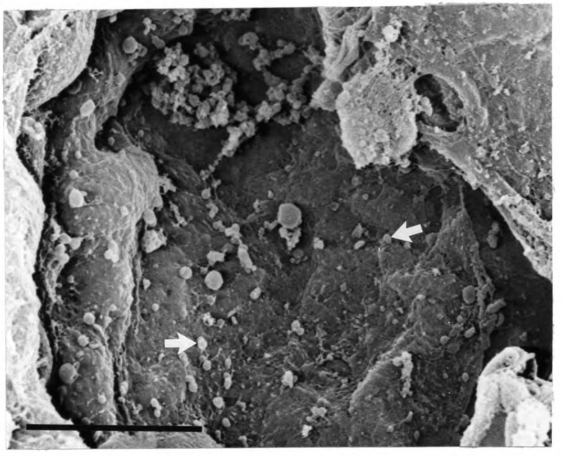

Figure 4-1. Scanning electron micrograph of a bovine lung explant 6 hours after incubation in minimal essential medium. Scattered Micrococcus spp. (arrows) are present in the alveoli, but degenerative changes in the alveolar epithelium are not evident. Bar = 10 micrometers.

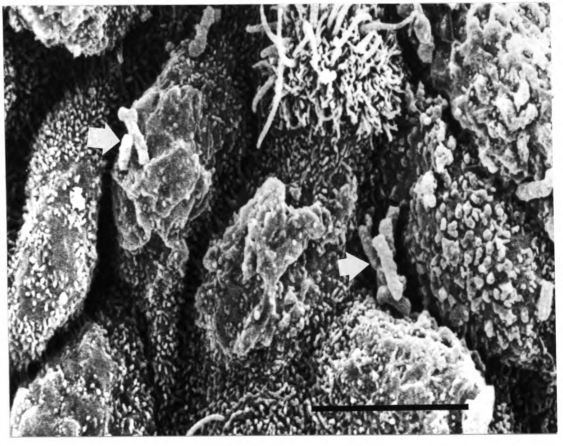

Figure 4-2. Scanning electron micrograph of a bovine lung explant after 2 hours incubation with living Haemophilus somnus bacteria. Only a few bacteria (arrows) are present on bronchiolar surfaces, and epithelial damage is not apparent. Bar = 5 micrometers.

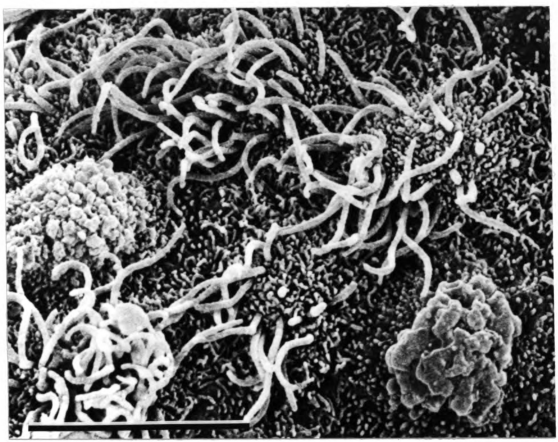

Figure 4-3. Scanning electron micrograph of the bronchiolar epithelium of a bovine lung explant inoculated with Haemophilus somnus 6 hours previously. No bacteria are visible in this area, and the cilia and microvilli of the epithelial cells are unaffected. Bar = 5 micrometers.

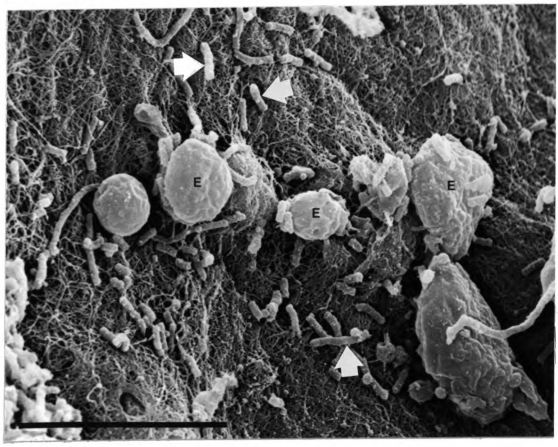

Figure 4-4. Scanning electron micrograph of alveoli with numerous bacteria (arrows) and detached epithelial cells (E) lying on the exposed fibrillar basement membrane. Bovine lung explant 6 hours after inoculation with Haemophilus somnus. Bar = 10 micrometers.

TABLE 4-1. Mean number (±S.D.) of Haemophilus somnus on 20-micrometer-square areas of bronchiolar and alveolar surfaces after in-vitro incubation of bovine lung explants with bacteria.

		Time after inoculation			
		1 hour	2 hours	4 hours	6 hours
ALVEOLI	1	3.2±1.30	8.4±1.52	86.2±26.29**	313.2±66.31**
	2	3.0±2.00	13.6±11.35	57.8±15.14**	333.6±64.22**
	3	7.4±2.41	8.8±4.44	40.4±11.67*	357.2±51.16**
	4	5.4±3.85	3.8±0.84	47.2±10.78*	377.0±33.64**
BRONCHIOLES	1	1.8±0.84	3.8±2.59	16.8±6.61*	13.0±3.67*
	2	2.2±1.30	2.6±1.82	45.8±10.57*	19.4±10.57*
	3	4.8±2.49	11.8±4.32	37.6±5.90*	50.6±11.63*
	4	2.6±1.52	2.6±1.14	27.8±8.17*	18.4±4.83*

** = mean is significantly higher (p = 0.01) than 1 and 2 hour alveolar means and all bronchiolar means.

* = mean is significantly higher (p = 0.01) than 1 and 2 hour bronchiolar means.

Note: When bacteria were too numerous to count, a value of 400 was assigned for statistical purposes.

extensive loss of alveolar epithelium (Figure 4-4).

In lung explants inoculated with 1 X 10^9 H. somnus and harvested one hour later, the numbers of bacteria on alveolar surfaces was not significantly different than the number of bacteria on bronchiolar surfaces.

DISCUSSION

Haemophilus somnus colonized alveolar locations in preference to bronchiolar sites in in-vitro lung explants maintained for a few hours following collection. Several reasons may be given for these observations.

The first is that H. somnus has little attraction for bronchiolar surfaces or surface materials when compared to alveolar surfaces or surface material. If this is true, then the lesions of bronchiolitis observed in natural and experimental H. somnus pneumonia are not directly related to the presence of H. somnus attached to bronchiolar epithelium. Rather, alternative hypotheses to bacterial localization on bronchioles are needed to explain the pathogenesis of H. somnus pneumonia.

A second explanation is that incubation in artificial fluid media alters cell surface components required for H. somnus-bronchiolar epithelial adhesion [7] or perhaps exposes normally covered alveolar surfaces. The alteration of surface structures seems a less likely explanation in lung tissue harvested from calves, a few minutes prior to inoculation with H. somnus, than in artificially cultured

cells of lung origin. [7] Also penetration of cell surface material is necessary for bacterial colonization. [9] The changes that might occur would likely be the removal of water soluble surface materials or the changing of hydrophobic or charged surface receptors. [5,7] The normal surface material of the alveoli is surfactant which acts as a water repellant. [18] Surfactant can, however, be washed from lung surfaces with any aqueous solution. [21] In cattle the terminal bronchioles, the short respiratory bronchioles and the alveolar ducts are probably also covered with a surfactant material. [15,16,24] The secretory bronchiolar epithelial cells of cattle, known as the Clara cell or non-ciliated bronchiolar cell, resemble the fetal or neonatal Clara cell of many other species. These cells have few secretory granules. [24] Although in many species the bronchioles and alveoli continue to transform and develop for several months after birth, [22] ruminant and swine lungs have a high degree of developmental maturity at birth. [1, 32] Calves, however, have not been specifically studied in this regard. It is likely that the bronchiolar surfaces of cattle are lined with alveolar derived surfactant. Mucus secreting cells are rare in the terminal bronchioles of cattle. [16]

In explants incubated for only one hour with the higher numbers of H. somnus, no differences in numbers of bacteria on alveolar versus bronchiolar surfaces could be demonstrated. This was similar to explants incubated with

bacteria for up to two hours. This suggests that if surface changes occurred to alter H. somnus localization, those changes probably occurred at the time the lung was placed in the medium and that time of incubation had little to do with localization of bacteria until after two hours. The hydrophobic and electrostatic bonds that may have been altered on the bronchiolar surfaces are not unique for H. somnus [5,7] and probably are active in adhesion of many bacteria. Therefore, it appears that H. somnus does not have any particular or unique prediliction for bronchiolar epithelium in preference to alveolar sites.

Although bronchiolar inflammation and damage were hallmarks of naturally occurring and experimental H. somnus pneumonia, there was no indication in this experiment that H. somnus selectively attached to bronchiolar epithelium in-vitro. These results rather suggest that the presence of H. somnus on bronchiolar epithelium in the early stages of experimentally induced H. somnus pneumonia is probably no more than the deposition of particles in terminal airways [8] and that the resultant bacterial cell to epithelial cell attraction does not give H. somnus any unique advantage over the host.

The ready colonization of alveoli after 4 hours incubation in this in-vitro model was somewhat surprising since alveolar lesion are inconsistently present in natural [2,3] and experimental disease. (Andrews, JJ: thesis, chapters 2 & 3) Perhaps normal in-vivo deposition and clearance

mechanisms ordinarily prevent any H. somnus reaching alveoli from remaining long enough to cause damage. The extensive degenerative changes in the alveolar epithelium inoculated in-vitro with H. somnus suggest that H. somnus or its products may be toxic for type I pneumocytes. The detachment of alveolar epithelium in inflammatory lung disease has been attributed to proteolytic enzymes (especially elastase) released by activated neutrophils. [4,27] Neutrophil elastase and other proteases are inhibited by alpha-1-antiprotease normally found in bronchoalveolar lavage fluids. [27] This enzyme may have been removed from surface locations in this explant model. Neutrophils were not observed, however, on alveolar or bronchiolar surfaces in the explants. The production of proteolytic enzymes by H. somnus has not been reported, [10,28] but production of proteases by similar bacteria, Pasteurella hemolytica [23] and Haemophilus pleuropneumoniae [14] has been documented. Endothelial cell death with detachment occurred in arterial organ cultures [30] and cultured endothelial cells [13] inoculated with H. somnus. The presence of a toxin was suggested to explain these observations. Supernatants of H. somnus growth, however, did not produce endothelial cell changes. [13] It is also possible that H. somnus growth in MEM alters the medium and depletes components necessary for pneumocyte survival in this artificial environment.

Another explanation for the localization of H. somnus on alveolar surfaces in this in-vitro explant model is the

deposition of organisms in the cup-like structures of the alveoli in preference to other sites due to the mixing motion caused by the orbital shaker and the gravitational settling of the bacteria. Lung blocks from a replicate of this experiment, performed without the orbital shaker, had few bacteria on bronchiolar surfaces while alveoli were well colonized. This suggests that gravity alone is not responsible for the localization of bacteria on alveolar surfaces in this model.

Naturally occurring and experimentally induced H. somnus pneumonia in cattle is characterized by a neutrophilic bronchiolitis often progressing to necrosis of bronchiolar mucosa (Andrews JJ: thesis, chapters 2 & 3). [8,3,11] While alveolar exudation is common in both the experimental and natural disease, necrosis of alveolar wall is uncommon (Andrews, JJ: thesis, chapters 2 & 3). [2,3] Neutrophils accompany both the alveolar and bronchiolar exudates and may contribute to epithelial and endothelial cell injury. [4,17] Acute lung injury induced by bacterial agents has generally been studied in living animals where it is difficult to separate the specific contribution to the disease process of different bacterial virulence factors or harmful effects of the host's reaction. Tissue cultures of lung epithelium and lung explants are therefore useful approaches to differentiate bacterial from host contributions to lung injury and to separate direct from indirect effects of toxins.

Isolated cell cutures of embryonic bovine lung have been used to detect the cytotoxic effects of various bacterial toxins including P. multocida type D rhinitis toxin. [25,26] Although this seems suitable for cytotoxin studies, isolated cell cultures are likely to have altered cell surface receptors and cytologic alterations which may not reflect the normal lung epithelium [7] and may produce erroneous information regarding bacterial attachment. Lung tissue explants maintained for several hours provided an in-vitro system that closely approximated in-vivo lung structure. It is essential, however, that explant systems be monitored and carefully controlled to differentiate between artifacts induced by culture techniques and the abnormalities induced by the bacterial agent.

Based on this study we concluded that the purulent bronchiolitis characteristic of natural and experimental H. somnus pneumonia cannot be explained based on selective attachment and localization of the bacteria on bronchiolar surfaces. These data also suggest that colonization of alveolar epithelium with H. somnus has direct detrimental effects on epithelial cells in the absence of neutrophils.

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CHAPTER FIVE

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Several discoveries characterize these studies. The first is the documentation of the dependence of the extent of pneumonia on the bacterial dose following intratracheal exposure of calves. Although this relationship has been assumed to exist, based largely on information from other less expensive laboratory animals, specific studies documenting this relationship in cattle have not been previously published. The dose-dependent model of H. somnus pneumonia reported in this thesis was reproducible, and the amount of pneumonia resulting from a given inoculum was predictable. The experimental pneumonia closely resembled that observed in the natural disease. The use of probit analysis to predict a mid-range effective dose should be useful in selecting dosages of H. somnus in experiments, utilizing this calf model, designed to measure changes in pneumonia production following treatments such as vaccination.

A second discovery supports a relatively new concept of the development of pulmonary inflammation. Bronchiolar exudates should not be viewed as extensions or movement of alveolar exudates into the tracheobronchial tree. Rather bronchiolar exudation is in part from post-capillary venules found in the bronchiolar submucosa. This venular exudation may respond to mediators of inflammation in a different manner than the alveolar capillaries thus resulting in different patterns of exudation into these two

different anatomical sites.

A third discovery was the intimate manner in which neutrophils in the bronchioles contact epithelial cells. This positions the inflammatory cell in a prime location to induce epithelial damage via release of oxygen metabolites and proteases. This close contact of neutrophils with the bronchiolar epithelium has not been previously reported as a feature of bacterial pneumonias in calves but may not be a unique feature of H. somnus pneumonia. Experiments designed to determine if this close neutrophil-bronchiolar epithelium relationship develops in other bovine bacterial pneumonias would help clarify this. The mild bronchiolar damage observed on the surface of the epithelial cells at 72 hours post-exposure suggests that neutrophils probably play an important role in mediating these changes. More severe necrosis of bronchiolar and alveolar exudates and tissues were invariably associated with the presence of high numbers of both bacteria and neutrophils. This supports the observation by others that H. somnus-induced cytotoxicity coupled with neutrophil induced damage may play a role in the development of the necrosis.

A fourth observation of importance was that, although Haemophilus somnus bacteria were present in contact with bronchiolar epithelium 1 hour post-exposure, selective attachment to these sites did not appear to be an important mechanism for the development of the bronchiolar lesions.

VITA

The author was born in Oskaloosa, Iowa in 1944. He attended primary school in North English, Iowa and secondary schools in Panora, Iowa and United Community Schools in rural Iowa near Boone. He graduated from high school in 1962 and entered the pre-veterinary curriculum at Iowa State University that same year. After completing the Doctor of Veterinary Medicine degree in 1968, the author joined a pet animal veterinary practice in Torrance, California. In 1969, he became a teaching assistant in the Department of Pathology, Michigan State University, while concurrently pursuing a Master of Science degree in pathology. In 1970, the author became a staff member of a mixed pet and farm animal practice in Perry, Iowa. In late 1970, he joined the faculty of the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Iowa State University as an instructor. The requirements for the Master of Science degree were completed and that degree was granted by Michigan State University in 1973. Since that time, the author has served as an Assistant Professor, Associate Professor and Professor of Veterinary Pathology at Iowa State University and as an Associate Professor of Pathology at the University of Missouri. In 1978 he was appointed pathology section leader of the ISU Veterinary Diagnostic Laboratory and currently continues to serve in that role. The author is a member of Phi Zeta, Gamma Sigma Delta, Alpha Zeta and ISU men's leadership honorary, Cardinal Key.