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Immunobiological properties of Haemophilus somnus

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Immunobiological properties of

Haemophilus somnus

by

Randall Duane Hubbard

A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of the
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MASTER OF SCIENCE

Department: Veterinary Microbiology and Preventive Medicine
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Signatures have been redacted for privacy

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**SECTION II. EFFECT OF HAEMOPHILUS SOMNUS FRACTIONS ON BOVINE POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS**  
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Haemophilus somnus (H. somnus) is the organism responsible for a variety of disease syndromes of cattle which have been termed the "H. somnus complex." Among these is the usually fatal, septicemic disease infectious thromboembolic meningoencephalitis (TEME), pneumonia, abortion and infertility. The organism is widespread in the cattle population where it infects dairy cattle and range cattle but is most prevalent in feedlot cattle.

H. somnus has been identified and studied for over 25 years but effective methods of prevention, treatment and control of H. somnus caused disease syndromes have not been developed. The organism is still a prevalent pathogen and responsible for considerable economic loss for the cattle industry. H. somnus is considered by some researchers to be the number one bacterial pathogen of feedlot cattle in some parts of the United States.

The bacterial-host interactions in the pathogenesis of disease and immune response to H. somnus infection are still poorly understood. Accumulation of neutrophils at the site of infection is a common characteristic of H. somnus disease, yet the infection is not controlled. Experiments were undertaken to determine whether H. somnus was able to interfere with normal neutrophil killing mechanisms. This thesis describes the extraction, partial characterization and effect of two factors from H. somnus which suppress normal neutrophil function indicating that these factors may
play a role in the pathogenesis of the disease.

Serologic studies of antibodies against *H. somnus* have been accomplished using numerous techniques including gel diffusion, bacterial agglutination, latex agglutination, passive hemagglutination, complement fixation and enzyme-linked immunosorbant assay (ELISA). Recent studies indicate that *H. somnus* antigens cross react with those from a number of other bovine pathogens. The high sensitivity of the ELISA procedure and the ability to utilize *H. somnus* specific antigens make this test an attractive choice for detection of anti-*H. somnus* antibodies. An ELISA test has been developed and is described in this paper utilizing phenol extracted lipopolysaccharide (LPS) as a specific *H. somnus* antigen for use in the test. Specificity of the LPS antigen was tested using rabbit antiserum to a variety of common bovine pathogens. This ELISA test was used to survey two herds for *H. somnus* antibody before, during and after a natural infection and the response to vaccination with two vaccines. These studies were undertaken to examine the specific antibody response to *H. somnus* that would not be confused by detection of antibodies to cross-reacting antigens of other microorganisms. Also, *H. somnus* LPS may play a role in the infective process and the detection of antibody specific for LPS may lead to a better understanding of the pathogenesis of the disease syndromes.

Examination of both cellular and humoral aspects of host resistance was conducted to increase our understanding of *H. somnus*–host interaction. The experiments described in this thesis relate to
these two different aspects of the immunobiological response to _H. somnus_. The results of this research will be described in two separate sections in manuscript form.
Historical Background of *Haemophilus somnus*

Infectious thromboembolic meningoencephalitis in cattle was first reported in 1956 by Griner et al. (54). Later, in 1960, Kennedy et al. reproduced the disease in calves with a "*Haemophilus*-like organism" (73). They described the organism as a pleomorphic, microaerophilic, gram-negative bacterium. Bailie et al. (7) reported TEME in Kansas feedlot cattle and described the presumed pathogen isolated as a small gram negative rod characterized as *Actinobacillus actinoides*-like." In 1969, Bailie (6) proposed the name *Haemophilus somnus* on the basis of its morphological, biochemical and cultural characteristics and the association of the bacterium with sleeper syndrome (TEME) of cattle. The true genus of this organism has not been established. *H. somnus* does not have a strict requirement for either X or V factor, a requirement for inclusion into the genus *Haemophilus* (8,75,139,158), but *H. somnus* does have a DNA guanine plus cytosine ratio within 1% of *H. influenzae b* and is therefore *Haemophilus*-like; hence the name has persisted. In 1968, Panciera et al. (104) described the disease state more fully as a septicemia. They described three separable but frequently overlapping clinical disease syndromes involving the central nervous system, the respiratory system, and the joints. The various disease syndromes and
their clinical signs were grouped together and referred to by Brown et al. (14) as the "H. somnus complex" in 1970. Included in this complex of disease syndromes was the peracute infection with clinical signs of fever, prostration, stiffness, CNS disturbance and sudden death due to TEME with hemorrhagic lesions in the brain, muscles, respiratory tract, intestine and kidney and cloudy fluid in the stifle joint. The acute infection with clinical signs of fever, depression, dyspnea, excessive lacrimation, nasal discharge, stiffness, soreness and nearly 90% morbidity, showed hemorrhagic lesions in the same organs as the peracute infection, accompanied by acute pneumonia in the lungs, laryngitis and peritracheal hemorrhages. The chronic state was described with the clinical signs of a dry hacking cough, lameness, stiffness, knuckling at fetlock, poor performance but usually low mortality. H. somnus is also recognized as a major cause of bronchopneumonia in calves (2,119) and endometritis in cows (26,28). H. somnus has been associated with preputial infections of bulls (28), infertility (27), abortion (25,119,144), weak calf syndrome (147), and mastitis (57).

H. somnus is distributed worldwide but is most prevalent in temperate climates. It has been isolated in many areas in the United States (7,24,73,104). H. somnus is a common and very serious pathogen of cattle in Canada (39,82,85,86,119,144). It has also been isolated in Germany, Italy, Scotland and Switzerland (139), United Kingdom (108,109,111) and Japan (156).

H. somnus is primarily a cause of disease in feedlot cattle
It may infect dairy cattle or range cattle as well
(104,119,131) and also both cows and calves in cow-calf herds (30).

The economic loss due to *H. somnus* infection is very great (119).

*H. somnus* has been isolated from the nasal cavities
(14,26,30,55,118) and tracheas (29) of healthy cattle. Corstvet et
al. (29) considered *H. somnus* as part of the transient, and possibly
indigenous flora of the bovine respiratory tract, with isolation rates
relatively constant between normal animals and animals with
respiratory disease and between seasons. The organism was more
frequently isolated in animals in which TEME occurred. Crandell et
al. (30) isolated *H. somnus* from the nasal cavities of calves
indicating early infection of calves in a cow-calf herd. The organism
persisted in the nasal cavities of these calves for up to nine weeks.

Horizontal transmission to nasal cavities of non-carrier calves
occurred when both the carriers and non-carriers were exposed to
infectious bovine rhinotracheitis (IBR) virus (30). A large cultural
survey has demonstrated a low overall prevalence of the organism in
the nasal cavities of cattle (118). After experimentally induced
septicemia and TEME, *H. somnus* could not be isolated from the nasal
cavity (138,153). The organism was not isolated from animals in
contact with other animals with *H. somnus* septicemia (16).

*H. somnus* has been isolated in high numbers from male
reproductive tracts or semen of healthy animals (26,52,65,69,147). *H.
somnus* was isolated from the prepuce in 26 of 31 normal bulls tested
(68). The organism was also isolated, in lower numbers, from the
urinary bladder and accessory sex glands of normal animals (67). Corboz (26) stated that the bull seems to represent one of the most important reservoirs of \textit{H. somnus}. Most isolations from the reproductive tract of bulls are from the prepuce. The prepuce has been shown experimentally to support the growth and survival of pathogenic strains of \textit{H. somnus} without loss of virulence (67).

\textit{H. somnus} has also been isolated from the female reproductive tract (26,90,147). \textit{H. somnus} had postnatally colonized the genital tracts of both male and female calves in a closed herd of asymptomatically infected cows (65). Urinary excretion has been suggested as a means of transmitting \textit{H. somnus} disease (101) and has been found to be a means of environmental dissemination in both natural and experimental cases of TEME (14,119,138,153). The high prevalence of \textit{H. somnus} in the urogenital tract of cattle suggests this site as an effective reservoir from which spread of \textit{H. somnus} by ingestion, inhalation or venereally may occur.

**Characteristics of \textit{Haemophilus somnus}**

\textit{H. somnus} bacteria are small, pleomorphic coccobacilli. They occur in both short chains and filamentous forms (27,48,73). Pleomorphism is reduced by \textit{in vitro} passage (73,104). \textit{H. somnus} is non-motile, non-sporeforming and non-piliated (6). Recent reports (20,26) suggest \textit{H. somnus} consists of a group of genotypically related
organisms with variable antigenic and biochemical characteristics.

**Staining reactions**

*H. somnus* is a Gram-negative non-acid fast organism which often demonstrates bipolar staining (6,27,144).

**Colony morphology**

Under optimal growth conditions typical *H. somnus* colonies are convex, circular, translucent, moist, glisteny and entire. Colonies are pin-point size after 24 h and 1-2mm after 2-3 days of growth. Older colonies have a granular appearance, become opaque, flatten at the edges and develop papillate centers (73,144). Hemolysis on blood agar has been reported, (48), as have colonial variants. Three colonial variants have been described as translucent, small opaque and large opaque by one group after egg embryo inoculation (98,99). Another group described four colonial variants as smooth, mucoid, intermediate and rough (26,28).

**Ultrastructure and encapsulation**

The cell envelope of *H. somnus* has been shown to consist of an outer membrane, peptidoglycan layer and inner cytoplasmic membrane, as in other gram-negative organisms (6,28,137).

Encapsulation was reported for one isolate, strain 8025, using light microscopy (91,153). Other investigators, using light microscopy, were not able to detect a capsule (6,26,48,101). Subsequent EM studies to demonstrate a polysaccharide capsule and negative staining to demonstrate pili have failed (137). EM studies to demonstrate pili on *H. somnus* organisms adherent on endothelial
cells in tissue culture have also failed (143). Definitive examination of \textit{H. somnus} for the presence of a capsule \textit{in vivo} has not been done. The fungi \textit{Cryptococcus neoformans} and the bacterium \textit{Yersinia pestis} have been shown to acquire capsules only after they have infected a host (34). Perhaps \textit{H. somnus} behaves in a similar manner.

**Pigmentation**

\textit{H. somnus} typically exhibits a yellow pigmentation. The color is most evident when many cells are pelleted together. It can also be seen when colonies are raised from the agar on a bacteriological loop (153). Pigmentation differences between colonial variants have been reported (26). Different quantities of a water soluble yellow pigment has been demonstrated between strains. Maximum absorption of the pigment occurred between 430-435 nanometers (136).

**Biochemical characteristics**

Reported biochemical reactions of \textit{H. somnus} have been varied. This is probably due to the various techniques used to study the reactions and not to variability of the organisms (28,48,134). \textit{H. somnus} produces cytochrome oxidase, reduces nitrates (6,48), acidifies litmus milk (48), produces indole, produces hydrogen sulfide, and ferments glucose (6,48). \textit{H. somnus} is negative for gelatin liquefaction (48), lecithinase production (123), urease production, citrate utilization (6,48), arginine dehydrolase production (48), methyl red/Voges-Proskauer reaction (6,48), lysine decarboxylation, ornithine decarboxylation (48) and growth on MacConkey agar (136).
Most strains ferment maltose, fructose, xylose, mannose, levulose, trehalose (6,10,48), sorbitol and mannitol (10,48). Most strains examined by Biberstein (10) fermented galactose and sucrose while all strains examined by Garcia-Delgado et al. (48) were negative or doubtful. *H. somnus* is negative for the utilization of dulcitol, lactose, raffinose, saccharose, rhamnose, salicin, arabinose and inositol (6,10,48).

**Growth requirements**

*H. somnus* is a fastidious microaerophile. Optimum growth was reported with brain heart infusion (BHI) supplemented with 10% bovine serum and 0.5% yeast extract (52), BHI agar with 10% bovine blood and 0.5% yeast extract (48) and on cystine heart agar with 10% bovine blood and 0.5% yeast extract (123). *H. somnus* grows well in the yolk sac of embryonated eggs providing a good medium for culturing and storing the organism at -70 C (48,104,147). *H. somnus* will grow independent of both X and V factors (10,123) and has been shown to synthesize porphyrins from amino levulinic acid (10). *H. somnus* is therefore different from other established *Haemophilus* sp. The enhanced growth of *H. somnus* in medium supplemented with "Isovitalex" (BBL Microbiology Systems, Cockeysville, MD.) led to the discovery that thiamine mono-phosphate (TMP) or co-carboxylase was a growth requirement (3). By measuring turbidity of BHI broth supplemented with TMP it was reported that 7 of 10 strains tested had an absolute requirement for co-carboxylase (3). Stephens et al. (135) recently reported the growth of the organism in BHI broth supplemented with
soluble starch, L-aspartate, tris(hydroxymethyl)aminomethane (THAM) and TMP, without serum or blood.

Optimal growth occurs when *H. somnus* is incubated in air supplemented with 10% carbon dioxide at 37°C (48) but equal growth at 5%, 10% and 20% carbon dioxide has been reported (123). No growth at 24°C or 47°C, moderate growth at 30°C and 43°C and optimal growth at pH 7.8 was also reported (123). Recent reports have indicated that some strains of *H. somnus* grow in ambient air (21). Isolates are reported to adapt to aerobic conditions after passage in artificial medium (73,144).

**Antibiotic sensitivity**

*H. somnus* is susceptible to most antibiotics (48,73,144). Using minimal concentrations of antimicrobial agents, 33 isolates were highly sensitive to penicillin G, ampicillin and novobiocin (140). Some isolates have been reported to be resistant to sulphonamides, chlortetracyline, lincomycin, bacitracin, streptomycin, penicillin, neomycin, polymyxin B, oxacillin, spiramycin and chloramphenicol (6,48,73,144). Because *H. somnus* responds well to antibiotic therapy, isolation of the organism from treated animals is unlikely.

**Immunological Properties of Haemophilus somnus**

**Serological diversity**

Originally, most *H. somnus* strains were thought to be antigenically identical (37,48,109,123). Recently, however,
serological diversity among 46 strains of *H. somnus* has been demonstrated using cross absorption agglutination tests between Swiss and American isolates (21). A common antigen associated with all isolates was reported as well as an American and a Swiss unique antigen. Utilizing a gelelectrophoresis-derived enzyme linked immunosorbant assay (GEDELISA) Corboz (26) demonstrated antigenic differences among colonial variants of the same strain in antigenic proteins of less than 15,000 MW.

**Serological cross-reactivity**

A variety of serological techniques have demonstrated antigenic relationships between *H. somnus* and a spectrum of other bacteria. Using bacterial agglutination tests *H. somnus* has been shown to cross-react with *H. ovis* (73,91,123), *Actinobacillus lignieresii* (48,91), *Listeria monocytogenes* (91), *Campylobacter fetus* (91), *Streptococcus agalactiae* (91), *Brucella abortus* (123), *Bordetella bronchiseptica* (123), *Yersinia enterocolitica* and *Mycoplasma bovis* (48). Using the same test other authors were not able to demonstrate antigenic cross-reactivity of *H. somnus* with *Actinobacillus actinoides* (91,123), *B. bronchiseptica* (48,91), *Pasteurella multocida, P. haemolytica, A. equuli, B. abortus, Neiseria catarrhalis* (48), *Staphylococcus aureus, Salmonella dublin* and *E. coli* (91).

Using a hemagglutination test, Miller et al. (91) tested the antigenicity of five *H. somnus* preparations in rabbits. Whole cells, sonicate, crude polysaccharide (formalin extract) and protein antigens were strongly antigenic. The fifth antigen, purified polysaccharide,
was weakly antigenic. The antigenic preparations also showed extensive cross-reactions with antibodies to antigens of *A. actinoides, A. lignieresii, B. bronchiseptica, B. abortus, E. coli, S. dublin, S. agalactiae, L. monocytogenes* and *Corynebacterium pyogenes*. Using a complement fixation (CF) test Dierks et al. (37) demonstrated weak relationships between *H. somnus* and *A. actinoides, M. bovis*, and various *Haemophilus* sp. including *H. agni, H. aegypticus, H. aphrophilus* and *H. parainfluenzae*. No cross reactions were reported using an immunodiffusion test (48).

Canto et al. (21) reported cross-reaction only with *H. agni* using whole cell or saline extracts of *H. somnus* in an ELISA test, but noted cross-reactions with *P. multocida, P. haemolytica* and *H. agni* using an ELISA test with sonicated or heat extracted *H. somnus* antigens. Using anti-*H. somnus* antiserum they found cross-reactions with the previous three organisms plus *S. dublin, S. agalactiae* and *C. pyogenes*. Stephens et al. (136) reported the cross-reaction of anti-*H. somnus* antiserum using an immunodiffusion test with antigens from *Histophilus ovis*, an Australian sheep pathogen that closely resembles *H. somnus, H. agni, H. haemoglobinophilus, A. seminis* and *A. lignieresii* but not with *H. influenzae*.

**Serological tests used to study *H. somnus***

The two most commonly used serological tests used to study *H. somnus* have been the bacterial agglutination (BA) and the complement fixation (CF) tests. These tests have been used to determine the immune response of animals (14,17,37,60,73,82,101,109), susceptibility
to experimental infection (17,37,55,138,153) and cross-reactions with other bacteria (37,48,91,138). Other methods have been a plate agglutination test (73), rapid slide agglutination test (109), passive hemagglutination test (91), immunodiffusion test (48), latex agglutination test (136) and ELISA tests (21,26).

Results using these techniques have been varied and sometimes contradictory. Serological surveys of normal cattle have shown 23-100% to be \textit{H. somnus} positive (60,68,138). Seroconversion has been noted in a herd of apparently healthy cattle indicating an inapparent infection (37,73). In herds with cases of TEME, 56-100% of the survivors were \textit{H. somnus} positive (37,60,73,101,108). Cattle with low titers were considered susceptible to intravenous challenge while positive or convalescent cattle were considered immune to challenge (17,37). Others have demonstrated an increasing antibody titer during the acute phase of the disease in all cattle that died while survivors did not show an increased titer (138).

Host Response to \textit{Haemophilus somnus}

The host response to \textit{H. somnus} has received limited study. Pennell and Renshaw (106) used whole cell, sonicate and crude polysaccharide (formalin extract) antigens as immunogens in cattle. Antigens were mixed with Freunds incomplete adjuvant. No response to the crude polysaccharide was detected but the sonicate, whole cell and protein antigens all induced the production of similar levels of
bactericidal and opsonic antibody as determined by in vitro tests. Both of these antibody effects were complement dependent. These results indicate that serum bactericidal and opsonic antibody, in conjunction with complement and leukocytes should protect against the septicemic spread of *H. somnus* infections. Simonsen and Maheswaran (127), suspecting animals succumbing to this disease may lack normal humoral defense factors, tested the in vitro bactericidal activity of serum from animals of different ages. They immunized four age groups with a killed vaccine in aluminum hydroxide adjuvant and demonstrated that the bactericidal activity of serum was dependent on heat-labile serum components (probably complement). This bactericidal activity was not passed to newborn calves. Calves, from 5 months to 1 year old, were the most susceptible age group for infection with *H. somnus* and were also the group with the lowest bactericidal activity. The serum from adult animals had the highest bactericidal activity inhibiting the growth of *H. somnus* by 60.9±16.9%.

Stephens et al. (138) measured serum antibody titers by seven serologic tests but found titers did not correlate with susceptibility to infection. They could not detect a response to a commercial *H. somnus* bacterin using a gel immunodiffusion (GID) test and this observation disagreed with the findings of Williams et al. (153) and Hall et al. (55) who detected a response to the bacterin with the (GID) test. Stephens et al. (138) found that cattle which were sero-positive when assayed by other tests, did not appear positive on the GID test. Stephens et al. (138) were able to reproduce disease signs
typical of naturally occurring TEME by IV inoculation of live *H. somnus*. They reported that previous exposure to *H. somnus* is necessary for typical TEME to occur, once infection is established. Inoculation of colostrum-deprived calves did cause septicemia but not TEME. These findings disagreed with those of Brown et al. (17) and Dierks et al. (37) who had previously reported that CF negative cattle were susceptible to IV inoculation of *H. somnus* and led to the development of TEME.

While *in vitro* and controlled studies indicate that vaccination of cattle with *H. somnus* bacterins would be beneficial this has not been apparent in field trials. The occurrence of TEME in vaccinated cattle is not unusual (86,118). Further field studies of *H. somnus* bacterins is needed to establish the efficacy of these products. Stephens (134) has isolated two outer membrane antigens that gave good antibody responses in cattle. One, an anionic antigen, gave good protection to IV challenge. Whole cell bacterins contain many antigens which have complex and possibly detrimental effects on the host immune system. Subcellular vaccines, such as Stephens anionic antigen, may someday be proved to induce protective immunity in the field.

Stephens et al. (138) speculated that, "The occurrence of bacteremia in the face of a high serum antibody titer indicates that the host phagocytic systems are no longer functioning effectively. Antibodies may react with the antigen in the blood to form antigen–antibody complexes that are not phagocytized, resulting in continually
increasing amounts of circulating antigen and immune complexes. This situation fulfills the requirements for a type III (serum sickness) hypersensitivity reaction."

**Pathogenicity factors**

In order for a microbe to be virulent, it must be able to invade its host and survive the defense mechanisms of the host designed to stop the infection. Pathogenicity of a microorganism is probably due to multiple factors including microbial characteristics that enable the organism to resist the host defense mechanisms (92). The mechanisms employed by *H. somnus* to invade the host and avoid the host defenses and establish a bacteremia are unknown. Simonsen (126) has shown, using an *in vitro* test, that only 55% of the organisms from either a virulent or an avirulent strain of *H. somnus* are phagocytosed by leukocytes. Antiserum to the virulent strain reduced the percent phagocytized. Stephens et al. (139) suggested that, since *H. somnus* is presumably an extracellular pyogenic organism, it must resist phagocytosis to spread throughout the body and cause disease. There is a need for more data on the ability of *H. somnus* to withstand phagocytosis and to define virulence factors of *H. somnus* (66).

Phagocytic cells occupy a central position in host defense against infection by microorganisms. Neutrophils are primarily responsible for the host defense against obligate extracellular pathogens through phagocytosis and destruction of these invading microorganisms. Many pathogenic microbes have survival mechanisms to protect against the killing processes of phagocytic cells, thereby
increasing the pathogenicity of these organisms. Mechanisms that protect the microorganisms may include avoidance of recognition, inhibition of chemotaxis, attachment, ingestion, oxidative metabolism or degranulation by neutrophils and possibly the elaboration of a leukocytic factor (35). Any of these inhibitors of neutrophil function could be considered as factors that increase the pathogenicity of the microorganism.

Neutrophil infiltration is a common feature in the lesions caused by *H. somnus*. In cases of abortion where *H. somnus* is implicated, abundant neutrophils are found in fetal airways (2,25,144). In cases of pneumonia, Andrews et al. (2) described microscopic lesions of peribronchiolar filling of alveoli with albuminous fluid and neutrophils. Neutrophils comprise the prominent inflammatory cell component of the bronchiolar exudate (2). Gross lesions associated with TME caused by *H. somnus* occur in the brain and irregularly in the spinal cord, mucosa of the esophagus, gastrointestinal tract and urinary bladder. Histological examination of these lesions reveals severe vasculitis, thrombosis, necrosis of vessel walls and intense neutrophil accumulation. These observations suggest that the bovine neutrophil is a likely candidate for an immunosuppressive effect caused by *H. somnus*. Such an effect could explain how *H. somnus* induces a disease state.
Enzyme-labelled Immunosorbent Assay for Detection of Anti-bacterial Antibodies

Sensitivity and specificity of serological techniques used to assay anti-\textit{H. somnus} antibodies in bovine serum have been questioned, especially in light of the numerous reports of serological cross-reactions with other bovine pathogens, using common serological assays (BA and CF) (21). The high sensitivity of the ELISA technique and the ability to select a specific antigen for high specificity make this test an attractive procedure for serological studies of \textit{H. somnus}.

Over the past 15 years, immunoassays, utilizing a sensitive indicator system to detect the specific antigen-antibody reactions, have gained great popularity. The ELISA technique, originally developed by Engvall and Perlmann (42), is an ideal diagnostic test because it is specific and sensitive. The heterogeneous ELISA technique, which employs a solid phase for attachment of specific antigen or antibody to specifically detect the inverse, has become very popular. The test has been adopted for the detection of antibodies to many microorganisms. These include many viruses from Adenovirus (11) to Rabies (4), parasites (32), fungi (100) and bacteria. Tests for detection of antibody to bacteria have been developed for Brucella (23,117), Escherichia (70,129), Mycobacterium (71,94), \textit{Neisseria} (15,50), \textit{Salmonella} (22,128), \textit{Staphylococcus} (155), \textit{Streptococcus} (116), \textit{Treponema} (145), \textit{Vibrio} (61), \textit{Yersinia} (23), \textit{Mycoplasma} (62), \textit{Rickettsiae} (56), \textit{Rhodococcus} (41) and \textit{Haemophilus}
(21,26). A battery of ELISA tests can be used to screen for antibodies to a number of agents in a diagnostic setting (100). The assay can be quantitative and used to detect specific immunoglobulin classes (4,22). The ELISA assay can be used to detect antibody at one serum dilution (105) or used to detect endpoint titers from two-fold dilutions (46,105). The ELISA test has been reported to be 10 times more sensitive than the CF test and nearly as sensitive as radio immunoassays (157).

Detection of Anti-lipopolysaccharide Antibodies in the Study of Bacterial Infections

Bacterial lipopolysaccharide (LPS) has been used as an antigen in the diagnosis of gram-negative infections. The Widal test is used to detect an LPS-specific antibody response to S. typhi in cases of typhoid fever (96). The detection of antibody to LPS has been used in the diagnosis of other bacterial infections caused by LPS-producing bacteria including brucellosis, tularemia, salmonellosis, shigellosis (95), infections due to Pseudomonas (110) and others. An increase in LPS-specific antibody titers during and following acute infection has been used to provide supporting evidence for the role of suspected pathogens (49,97). Detection of anti-LPS antibodies has been used to differentiate between single and mixed infections, relapse or reinfection, for the diagnosis of subclinical infection and for the clarification of epidemiologic aspects of disease (95).
The antibody response to LPS from a gram-negative organism can play a role in the outcome of an infection by that organism. The anti-LPS antibody can add to the pathogenicity of the LPS. For instance, it has been reported that antibody to LPS can enhance the toxicity of the lipid A portion of LPS (47). However, antibody against LPS is generally beneficial. Antibody against LPS can function as an antitoxin and as such can diminish the mortality from gram-negative bacteremias (13). *Pseudomonas* LPS has been used as an experimental vaccine for pulmonary protection from infection by that organism (107). Davis et al. (31) have reported that anti-meningococcal LPS antibody can neutralize the effects of meningococcal endotoxins. Experimental immunization of mice and chickens with LPS from rough *N. gonorrhoeae* protected against infection. Embryonated eggs from the immunized hens were also protected against infection (36). Antiserum to core LPS can prevent death from bacteremia in neutropenic rabbits when given therapeutically after the onset of bacteremia due to *P. aeruginosa, E. coli* and *K. pneumoniae* (13). High titers of antibody to core LPS at the onset of human bacteremia has been associated with significantly less frequent shock and death in patients with bacteremia due to gram-negative bacilli (160).

Other aspects of the humoral immune response to LPS have been studied. LPS has been reported to be a T-cell independent immunogen as demonstrated by antibody responses to *E. coli* LPS in thymusless nude mice (84). It has been reported that young children may respond less actively to antigenic stimulation by LPS of infecting
microorganisms but can mount a specific antibody response to the organism (49). Antibodies directed against O-polysaccharide components of LPS do not regularly elicit a protective response. Production of such antibodies, even in high titers, by patients with typhoid fever is frequently encountered and yet the disease continues and may be fatal (96). Similar observations have been made in subjects with urinary tract infections. Bacteria in the bladder and from the kidney may be coated with O-specific IgG, M, or A antibodies without termination of infection (96). A variety of tests have been used for quantification of antibody to LPS including ELISA (44,53,146) and passive hemagglutination (146).

Microbial Mechanisms of Resistance to Phagocytes

Two major microbicidal events occur inside the PMN when a microorganism is ingested (113). First, the generation of highly toxic products by the PMNs oxidative metabolism and, second, the enzymatic destruction and digestion of the ingested microorganism by the lysosomal enzymes present in the intracellular granules which fuse with the phagosome releasing these enzymes. Killing of ingested bacteria by neutrophils can be oxygen dependent or oxygen independent. The oxygen dependent system consists of myeloperoxidase plus halide plus hydrogen peroxide, one of the PMNs most potent killing mechanisms, or myeloperoxidase independent reactions with superoxide anion, hydroxyl radicals, singlet oxygen and hydrogen peroxide. The
oxygen independent mechanisms consist of the acid environment in the phagolysosome, lysozyme, lactoferrin, cationic proteins, lysosomal hydrolase, and neutral proteases (78).

The oxygen independent antibacterial systems have a more limited effect. One of the most abundant enzymes in the neutrophil, lysozyme, is very effective against sensitive microbes. Most gram negative bacteria however, are protected from the action of lysozyme by their mucopolysaccharide cell wall. Susceptibility to the bactericidal action of the contents of the primary granule is decreased as the lipopolysaccharide (LPS) chain of the cell wall increases in length (34). It is also postulated that LPS sterically hinders the binding of granule released proteins to the bacterial cell wall.

If the bacteria can be trapped in the phagosome it may die due to other factors. These include lack of nutrition and exposure to the acid environment in the vacuole (78). Lactoferrin in the phagosome can chelate iron that is essential for the growth of some organisms (103). Hydrolases and cationic proteins found in the granules also have antibacterial effects (59).

The oxygen dependent but myeloperoxidase independent systems are activated during the metabolic burst. Singlet oxygen, hydroxyl radicals, superoxide anion and hydrogen peroxide, the four by-products of the oxygen burst, have antimicrobial activity which is enhanced by the addition of myeloperoxidase and halide. Acting alone, the myeloperoxidase independent antibacterial substances work more slowly and without as broad an effect (5). Associated with the PMN oxidative
metabolism is the myeloperoxidase dependent iodination reaction. This reaction occurs inside the phagocytic vacuole via the action of hydrogen peroxide and myeloperoxidase from the primary granules in the PMN.

Interference with neutrophil activity may be an important aspect of the pathogenicity of a microbe. One possible mechanism would be if the microbe could circumvent recognition as foreign by the host immune system. This does occur in the case of adult schistosomes which acquire red cell determinants in the presence of those cells and thus are masked by these host cell antigens and not recognized as foreign. This mechanism explains why eosinophils are not effective against the adult worm (51). No examples of bacterial resistance by this mechanism are known.

Another mechanism of resistance may be to inhibit neutrophil chemotaxis. Delayed neutrophil arrival at the site of infection may permit the bacteria to establish an infection. Impairment of neutrophil migration has been demonstrated in the presence of S. typhi, Neisseria meningitidis, Pseudomonas aeruginosa or Serratia sp. (34), virulent strains of Brucella but not avirulent strains (40) and in virulent but not avirulent strains of Mycobacterium tuberculosis. Cord factor, a surface lipid from M. tuberculosis has been determined to cause inhibition of chemotaxis (1). A surface mucopeptide from virulent but not avirulent strains of S. aureus were found to be anti-chemotactic for mouse leukocytes in vitro (149). The mechanism of chemotactic impairment may be a failure to stimulate it or direct
inhibition of neutrophil motion.

There are some reports of bacterial infections which fail to stimulate chemotaxis. It has been shown that patients with gonococcal urethritis activate complement rapidly generating the chemotactic peptide C5a to stimulate neutrophil chemotaxis. Gonococci isolated from patients with disseminated gonococcal infection activate complement slower and thus stimulate less chemotaxis (34). P. aeruginosa uses a different tactic to impair phagocyte chemotaxis. It produces a protease that acts on complement components in fluid phase or cell bound Cl and C3. The inactivation of these complement components inhibits neutrophil chemotaxis and phagocytosis (122).

Another anti-chemotactic mechanism is employed by enterotoxigenic E. coli or V. cholera. Both of these organisms produce a substance which has a direct toxic effect on neutrophils. The toxin stimulates adenylate cyclase activity, increasing the intracellular cAMP levels. This inhibits neutrophil functions such as chemotaxis. The effect is enhanced with addition of phosphodiesterase inhibitors or dibutyryl cAMP (34).

Another method of microbial resistance to neutrophil function is repression of attachment and ingestion of the microbe by the phagocyte. Three proteins, the K-88 antigen of E. coli, the polyglutamic acid capsule of B. anthracis and the antiphagocytic fimbriae associated with the M protein of streptococci, inhibit attachment and ingestion of bacteria by phagocytic cells (34). Capsular polysaccharides are a common antiphagocytic mechanism of many
organisms which results in a need for specific anticapsular antibody for phagocytosis to proceed. These bacteria include \textit{N. gonorrhoeae} (58), \textit{N. meningitidis} (112), \textit{K. pneumonia} (130), \textit{S. aureus} (88), \textit{B. anthracis} (74), \textit{C. fetus} (87), \textit{Bacteriodes fragilis} (102), \textit{P. multocida} (83), \textit{E. coli} (64), \textit{S. pneumonia} and \textit{S. pyogenes} (45), \textit{H. influenzae} group B (141) and \textit{Y. pestis} (154). Capsular antiphagocytic mechanisms seem to involve blocking of binding either complement or antibody to the organism or masking bound opsonins and there is evidence for both hypotheses (34). The antiphagocytic activity is directly proportional to the amount of surface capsular material (64). Many bacterial pathogens produce a capsule that enables the microbe to resist attachment to and ingestion by host phagocytes.

Resistance to ingestion is another bacterial mechanism for survival. Evidence to support the localized decrease of neutrophil membrane fluidity inhibiting phagocytosis caused by virulent organisms has been published. Virulent but not avirulent \textit{N. gonorrhoeae} decreased membrane fluidity but did not interfere with ingestion of a second organism; however, mycoplasma attached to neutrophils interfered with killing of a second organism, \textit{E. coli} (34). Further study of the gonococci revealed that less primary granule release was stimulated by the virulent strains than by avirulent strains. This may be due to a failure of forming a phagosome containing the gonococci or a failure of primary granule-phagosome fusion (33).

Sawyer (120) has shown that influenza virus attachment to neutrophils depressed glycolysis and subsequent ingestion and killing
of bacteria. Several viruses depressed chemilluminescence of neutrophils but did not inhibit ingestion of zymosan. Degranulation is inhibited by some intracellular pathogens. Macrophages ingesting live *M. tuberculosis* did not undergo normal degranulation into the phagosome. However, ingestion of killed or opsonized *M. tuberculosis* did not inhibit degranulation, although the live, opsonized microbe survived despite degranulation. An anionic sulfatide has been isolated from the cell wall of *M. tuberculosis* that inhibits degranulation (18). Degranulation is also inhibited by an increase in cAMP as seen in the case of macrophages ingesting *M. lepraemurium* (34).

Resistance to microbicidal oxygen products can be achieved by a number of bacterial mechanisms. Catalase-rich strains of staphylococci had a better survival rate and decreased myeloperoxidase dependent iodination, probably due to destruction of hydrogen peroxide. The oxidative potential of singlet oxygen is quenched when in contact with substances with a large number of conjugated double bonds, e.g. carotenoids (34). Thus, a pigmented bacterium may be able to quench singlet oxygen produced during the oxidative burst (26).

The ultimate antiphagocytic mechanism is bacterial killing of the phagocyte. Streptolysins S and O, beta-hemolysins of *S. pyogenes* and *S. pneumoniae*, are toxic to PMNs, macrophages, lymphocytes and erythrocytes (63). Some staphylococci have a leukocidin which acts only on phagocytes, increasing membrane permeability and inhibiting degranulation (63). *P. aeruginosa* produces a cell bound protein
cytotoxic for PMNs but not for erythrocytes (121). This toxin also disrupts membrane permeability. The action of these cytotoxins can be neutralized by specific antibody.
RESEARCH OBJECTIVES

Disease syndromes caused by *H. somnus* are a major problem in the cattle industry. The objective of this research was to develop a better understanding of the host immune response to *H. somnus*. An enzyme-linked immunosorbent assay (ELISA) test was developed to measure the humoral immune response of cattle to *H. somnus*. Neutrophil function assays were employed to assay for pathogenicity factors associated with *H. somnus*. Experimentation reported in the thesis was concerned with these two aspects of the host immune response. Therefore the findings of this research are presented in two individual sections in manuscript form.

The study of the humoral immune response to *H. somnus* is not well understood. Workers, using various serological methods, have reported conflicting results. Serological cross-reactivity of *H. somnus* antigens with those of other common bovine pathogens has been demonstrated (21, 37, 48, 73, 91, 123, 136). The need for a sensitive, yet specific, serological method for the detection of antibodies to *H. somnus* is needed. The ELISA test has become a popular serological method used to detect antibodies to many microbes. Advantages of the ELISA include high sensitivity by incorporating a very sensitive enzyme catalyzed reaction as the indicator system, inexpensive reagents and equipment, potential for automation and the ability to use very specific antigens attached to the solid phase for great specificity. This research reports on the development of a specific
ELISA test for the detection of antibodies to *H. somnus* which utilizes a unique antigenic determinant, LPS of *H. somnus*. The detection of antibody to LPS, which may have a role in the pathogenicity of infection, may lead to a better understanding of the host humoral response to *H. somnus*.

In vitro methods to evaluate the functions of bovine neutrophils have been developed. These methods can be adapted to test for impairment of neutrophil function by the addition of bacterial fractions to the reaction mixture. The suppression of neutrophil function is a common virulence mechanism employed by bacteria to resist phagocytosis and killing. Lesions associated with *H. somnus* infections are characterized by neutrophil accumulation. The presence of infection in the face of neutrophil infiltration suggests that the neutrophils may not be functioning effectively. Humphrey and Stephens, in their excellent review of *H. somnus* (66), called for further studies to establish the nature of the host-parasite relationship and to define bacterial virulence factors of *H. somnus*. This research reports on the demonstration of two soluble, heat extracted surface factors that inhibit separate neutrophil functions. One factor, of less than 10,000 MW, has a suppressive effect on iodination of protein in the neutrophil, a key reaction in the potent myeloperoxidase-halide-hydrogen peroxide killing mechanism of the neutrophil. The second factor, of greater than 300,000 MW has a suppressive effect on phagocytosis of opsonized bacteria by the neutrophil.
SECTION 1.

DETECTION OF ANTIBODY TO *HAEMOPHIILUS SOMNUS* LIPOPOLYSACCHARIDE BY ENZYME-LINKED IMMUNOSORBENT ASSAY IN SERUMS FROM NORMALLY INFECTED AND VACCINATED CATTLE

Summary

A sensitive and specific enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of immunoglobulins to *Haemophilus somnus* lipopolysaccharide (LPS) in bovine serums. Titers from 0 to 1:10,240 were observed with this assay. This assay was useful in detecting specific antibody to an *H. somnus* antigen, LPS, with little cross-reactivity with antigens of other common bovine pathogens, as well as providing insight into the humoral response to natural *H. somnus* infection and vaccination. Low to moderate antibody titers were detected in the serum of both naturally infected and vaccinated cattle. No direct correlation between antibody titer and disease resistance was observed. Detection of antibody to *H. somnus* LPS may lead to a better understanding of the role of LPS in the pathogenesis of the disease syndromes caused by *H. somnus*.

Introduction

*Haemophilus somnus* (*H. somnus*) is the organism responsible for a
variety of disease syndromes of cattle, which have been termed the "H. somnus Complex" (14). Among these disease syndromes are infectious thromboembolic meningoencephalitis (TEME), septicemia, pneumonia, infertility, weak calf syndrome and abortion (2,7,14,25,26,27,28,54,73,104,108,118,144,147). The bacterium has been shown to be an inhabitant of the upper respiratory tract of healthy cattle (29) and has also been isolated from the normal reproductive tracts of cattle (27,28,65,69,82,118,144,147). Many healthy cattle have serum antibody against H. somnus although the proportion of responders in the cattle population varies with the serological test used. Hoerlein et al. (60) found 24% of over 2200 cattle had serum bacterial agglutination test titers of 1:25 or greater. Using the same test Stephens et al. (138) found 91% of 80 cattle had titers of 1:4 or greater. Dierks et al. (37) found 23% of 2000 cattle had complement fixation test titers of 1:8 or greater.

The pathogenic mechanism of infection by H. somnus, although not understood, has been thought by some researchers to be due to a lack of normal humoral defenses. Because of cross-reacting antigens of H. somnus (21) serological surveys may not reflect the true state of humoral immunity (139). As one part of an attempt to better understand the humoral response in calves a specific and sensitive ELISA test was developed to detect antibody to the lipopolysaccharide (LPS) of H. somnus. The ELISA test was used to monitor the humoral response to H. somnus LPS in a herd of calves undergoing a natural infection and in calves vaccinated with a commercial H. somnus
bacterin and a ribosomal H. somnus vaccine.

Materials and Methods

Antigen preparation

H. somnus strain 8025, initially isolated from bovine brain with lesions of TEME, was stored at -70 °C in egg yolk suspension (2 ml). An aliquot of this stock culture of H. somnus, in egg yolk, was used to inoculate 10 ml of brain-heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% normal bovine serum and 0.5% yeast extract (Difco), (BHISY), incubated for 12 h at 37 °C in an atmosphere of 5% carbon dioxide and 95% air, then used to inoculate 100 ml of the BHISY medium which was incubated for 24 h. Two ml of this broth culture were used to inoculate each Roux bottle containing the BHISY medium and 1.5% Bacto-agar (Difco). The Roux bottles were rotated by hand to spread the inoculum over the surface of the agar and the bottles were incubated for 48 h. The bacteria were harvested by adding 5 ml of 0.015 M phosphate buffered saline solution (PBS) to each bottle. The surfaces of the bottles were then scraped with a sterile wire and the bacterial suspension was harvested by aspiration and pooled. The cells were washed three times by alternate centrifugation and resuspension (10,000 x g for 30 min at 5 °C).

Cold phenol extraction of LPS

Cold phenol extraction of H. somnus cells was performed by a modification of the method of Westphal.
and Jann (152). Thrice washed cells were resuspended in sterile distilled water (DW) and mixed for 4-5 min at low speed with a Virtis homogenizer (Virtis Co., Gardiner, N.Y.), then for 4-5 min at high speed with a vortex mixer to uniformly suspend the cells. The homogeneous mixture was then stirred in a Waring blender (Waring Products, New Hartford, Conn.) for 2 min. To this mixture was then added 2.6 volumes of phenol and 2 volumes of DW. This suspension was reblended for 8 min. The mixture was cooled to 20°C and centrifuged at 3000 x g at 4°C for 10 min.

The upper aqueous phase was siphoned off and saved and the lower phenol phase was retreated with one half volume (of the lower phase) of DW and recentrifuged at 3000 x g at 4°C for 20 min. This second upper aqueous phase was again aspirated and added to the first aqueous phase and the mixture was recentrifuged to remove any particulate material. The supernatant fluid was dialyzed against four changes (200 x volume) of DW at 4°C for 4 days, clarified by centrifugation at 10,000 x g at 4°C for 20 min and then held at 4°C. One mg NaCl per ml and two volumes of cold (4°C) acetone were added to the supernatant material and the mixture was left standing for 1 h at 4°C to allow the LPS to precipitate with nucleic acids and other materials. The precipitate was removed by centrifugation at 4000 x g for 20 min at 4°C and then resuspended to the original volume in DW. Cold (-20°C) absolute ethanol was then added to a final concentration of 25% (v/v) and the mixture was allowed to stand overnight at 4°C. Particulate material (including the precipitated RNA) was removed by
centrifugation at $4000 \times g$ for 20 min at 4°C and saved.

Dry NaCl was added to the supernatant to a final concentration of 0.4 M followed by 8 volumes of cold (-20°C) absolute ethanol, added dropwise, and the mixture was allowed to stand for 1 h at 4°C. The precipitate (containing the LPS) was collected by centrifugation at $4000 \times g$ for 20 min at 4°C, allowed to dry for 6-8 h at 4°C and then redissolved in DW in the same volume as the dialyzed clarified supernatant. Any insoluble material was removed by centrifugation at $4000 \times g$ for 10 min at 4°C. The supernatant, containing the partially purified LPS, was dialyzed against three changes of DW (200 x volume) at 4°C for 72 h and then dispensed in aliquots and stored at -20°C. An aliquot was evaluated for dry weight, percent protein (2.28%) as determined by the Bio-Rad method (12) and percent neutral sugars (20.35%) as measured by the phenol-sulfuric acid method (38) with glucose used as a standard.

Endotoxic activity was determined by inducing endotoxin mediated primary skin reactions in rabbits by the method of Larson et al. (80), by chicken embryo lethality using the method of Smith and Thomas (133) and by induction of the local Schwartzman reaction in rabbits by the method of Kasai and Nowotny (72).

Preparation of other ELISA antigens A heat extract antigen (HE) of *H. somnus* was made by washing cells, grown as for LPS, three times with PBS. The cells were then diluted with PBS to a standard concentration containing 420 million cells per ml. This cell
suspension had an optical density of 0.4 O.D. at 600 nm when diluted 1:10 in PBS. This suspension was then heat treated at 60 C for 1 h with occasional agitation. The killed suspension was then centrifuged at 3000 x g for 30 min at 4 C, and the supernatant was used as an antigen for the ELISA assay.

An incubated extract (IE) of *H. somnus* was made by growing and standardizing the cells as was done for the HE antigen. This suspension was incubated at 37 C in an atmosphere of 5% carbon dioxide and 95% air for 18 h, centrifuged at 3000 x g for 20 min at 4 C and the supernatant used as an antigen for the ELISA assay.

A lysozyme extract (LE) of *H. somnus* was also prepared. A lysozyme buffer (35) containing 60 mM sodium chloride, 30 mM sodium citrate, 50 mM dipotassium phosphate and 150 ug/ml lysozyme (Sigma) pH 7.5 was used to treat the washed cells. An equal volume of the lysozyme buffer was mixed with a 10x of 0.4 O.D. (600 nm) suspension of washed *H. somnus* cells. The mixture was incubated at 37 C overnight (18 h). The mixture was then centrifuged at 3000 x g for 30 min at 4 C and the supernatant was used as an antigen for the ELISA assay.

A diethylene glycol extract of *H. somnus* was also prepared by a modification of the method of Morgan (93). Washed *H. somnus* cells were suspended in ten parts (w/v) of fresh diethylene glycol (Sigma) and shaken mechanically for three days at room temp. Methanol (5% v/v) was added to reduce the viscosity. The cells were removed by centrifugation at 3000 x g for 30 min at 4 C and the supernatant was
Figure 1. Preparation of ELISA antigens
dialyzed against three changes of DW (200 x volume) at 4 °C for 72 h and then used as an antigen for the ELISA assay.

Cattle

Two groups of mixed breed cattle, A and B, housed together, which were two months of age and newly weaned at the start of the experimentation were used. Group A consisted of 38 calves which were randomly assigned to one of three groups, those immunized with Somnugen (S), 12 calves, those immunized with an experimental ribosomal vaccine (R), 13 calves, and a non-vaccinated control group (C), 13 calves. Group B consisted of 63 calves that were not immunized with H. somnus preparations but were used to follow the humoral response to H. somnus LPS during natural infection. H. somnus was active in both groups. Several of the calves in group B died during the experimentation from confirmed H. somnus pneumonia. A third group of non-vaccinated, eight month old calves, that had previously shown clinical signs of H. somnus pneumonia, were also tested to compare their humoral response to H. somnus LPS with the response in the younger calves.

Vaccine preparation and vaccination protocol

The two antigens used for vaccinating cattle were a commercial bacterin, Somnugen (S) (Bio-Ceutic, St. Joseph, Mo.) and a ribosomal vaccine (R) prepared by a modification of the method of Smith and Bigley (132). H. somnus cells were grown and washed by the method for preparation of LPS except the cells were washed in cold (4 °C) PBS.
After the third wash, the cells were resuspended in a minimal amount of 0.01 M Tris-HCl buffer (Tris[hydroxymethyl] aminomethane) (Sigma) containing $5 \times 10^{-3}$ M magnesium chloride. The cells were disrupted in a French Press (courtesy of Dr. Williams, Dept. of Microbiology, ISU) by running the cells through a cold cylinder twice and collecting them in a cold slurry of Tris-HCl buffer. Cellular debris and whole cells were removed by centrifugation at 39,000 x g for 1 h at 5 C. The supernatant, containing the RNA protein, was brought to 0.1 M sodium concentration by the addition of sodium chloride and extracted with two volumes of cold (-20 C) 95% ethanol, added dropwise, while stirring with a magnetic stirrer at 4 C. The mixture was then left at -20 C for 24 h to allow precipitation of nucleic acids and proteins, followed by centrifugation at 10,000 x g for 10 min at 5 C. The supernatant was discarded and the precipitate was allowed to dry by evaporation for 3 days at -20 C. The precipitate was resuspended in a minimal amount of Tris-HCl buffer and stored at -70 C. An aliquot of this material was evaluated for dry weight, protein concentration (6.1 ug/ml) by the Bio-Rad method (12) and for carbohydrate content (10 ug/ml) by the method of Dubois et al. (38).

Group S was given Somnugen according to manufacturers specifications. Two doses were given, the first on day one and the second at the time of the second bleeding two weeks later. Group R was given one dose of the ribosomal vaccine on day one. The control group, C, was not treated.
Serum samples

Serum samples from the vaccinate group and the control group were collected before immunization and twice more at two week intervals. Serum samples were obtained at weaning and were continually collected at two week intervals from an untreated herd in an effort to monitor serum antibody levels in a natural situation. Serums from a third group of calves that were recovering from a session of respiratory disease were collected twice at a three month interval. All serums were kept frozen at -40 C until assayed for antibody against \( H. \) somnus LPS.

ELISA for detection of antibodies to \( H. \) somnus in rabbit serum

An ELISA test for the detection of anti-\( H. \) somnus antibodies in serum from immunized rabbits was developed for the detection of cross-reactive \( H. \) somnus antigens with those of other common bovine pathogens using antisera to eight common pathogens. The common pathogens used for immunization were strains of \( S. \) agalactia, \( B. \) bronchiseptica, \( A. \) lignieresii, \( L. \) monocytogenes, \( C. \) fetus, \( C. \) pyogenes, \( Moraxella \) bovis and \( H. \) somnus.

The following microorganisms were injected as viable cells: \( A. \) lignieresii, \( S. \) agalactia, \( C. \) fetus, \( B. \) bronchiseptica, and \( H. \) somnus. For this purpose, these organisms were grown for 18 h on 10\% bovine blood agar (enriched with yeast-extract-BHI base) then washed off the plates with sterile PBS, washed twice by centrifugation and resuspension in PBS and standardized to the density of McFarland
nephalometer tube #4.

*L. monocytogenes*, *C. pyogenes* and *M. bovis* were grown as above and then a bacterin was prepared as follows: The cells were harvested and washed three times in formal-saline (0.3% of commercial 40% formaldehyde) and reconstituted in 10 ml of formal-saline. This stock suspension was incubated for 48 h at 37 C in an atmosphere of 5% carbon dioxide and 95% air and then stored at 4 C. A portion of this stock solution was diluted with formal-saline to a density equal to tube #4 of a McFarland nephalometer and used for immunization. The immunization schedule and doses for both the viable cell preparations and the bacterins were to give 1 ml at multiple subcutaneous sites on the back of rabbits on day 1, 4, 8 and 11. A 2.0 ml dose was given on days 15 and 19. The rabbits were then sacrificed on day 23 and the serum collected.

Antiserum to *Pasturella multocida* was prepared by Mr. Hyoik Ryu, Dept. of Veterinary Microbiology and Preventive Medicine, I.S.U. The organisms were grown in Roux bottles on starch-dextrose agar after an inoculum was prepared in the BHISY medium. The Roux bottles were incubated for 24 h at 37 C and the cells harvested in PBS, centrifuged and an aliquot was used to determine the dry weight of the suspension. Five mg/ml (dry weight) of the whole cell antigen was mixed with an equal amount of Freunds complete adjuvant. One ml was given at various sites on the back of rabbits. The rabbits were boosted three times at one week intervals with 1 ml of the same amount of antigen in Freunds incomplete adjuvant at various sites on the back of rabbits as
before. The rabbits were bled out two weeks after the last booster injection. Antiserum to *Pasturella hemolytica* was provided by Dr. Glynn Frank, National Animal Disease Center, Ames, Iowa.

**ELISA procedure**

Antibody to *H. somnus* lipopolysaccharide was quantitated by using a modification of the enzyme-linked immunosorbant method of Engvall and Perlmann (42). Polyvinyl chloride (PVC) plates (Dynatech Laboratories Inc., Alexandria, Va.) were coated with 100 ul (10 ug/well) of phenol extracted LPS in sodium carbonate-bicarbonate buffer pH 9.6 and were incubated for 3 h at 37 C in a humid incubator and then overnight at 4 C. The optimum antigen concentration was determined by a checkerboard assay. Plates stored at 4 C in air-tight plastic boxes under humid conditions retained stable activity for up to 1 month. At the time of use the LPS antigen solution was decanted and the plate was washed six times with wash solution (WS) pH 7.2 containing 0.5 M sodium chloride and 0.5% Tween 80 (w/v) using a Skatron plate washer (Flow Laboratories Inc. McLean, Va.).

Dilute normal horse serum (100 ul of 3% in WS (v/v)) was added to each well and incubated at room temperature for 30 min to fill any open antigen binding sites in the PVC plate wells. Plates were then washed as before and 100 ul of 3% ovalbumin in WS (w/v) was added to each well. Bovine antiserum was diluted 1:2 with 0.05M dithiothreitol (DTT) (Sigma) and incubated for 30 min at 37 C to dissociate the IgM pentamer which has been reported to non-specifically bind to LPS (79).
This mixture was then diluted 1:5 with 3% ovalbumin in WS. The outer wells of the PVC plate were not used for ELISA because they gave varying results which left 60 wells in the center of the plate for use. The plate was divided into 10 columns of six rows allowing for titration of 9 unknown serums and one known positive or negative serum. To the first row of each column was added 100 ul of the DTT treated and diluted antiserums. Two-fold dilutions were then quickly made down the rows using a multichannel micropipettor (Flow Titertek 12 channel, Flow), and the plate was incubated, covered, at 37 C with gentle shaking for 20 min, the optimum time having been previously determined. The plates were then washed as before, followed by addition of 50 ul of peroxidase conjugated antiserum to each well, the appropriate dilution having been previously determined by a checkerboard assay. Affinity purified goat anti-bovine IgG light and heavy chain specific peroxidase conjugated antiserum, KPI, (Kirkegaard & Perry Laboratories Inc. Gaithersburg, Maryland), was used for detection of anti-\textit{H. somnus} LPS antibodies in bovine serum while peroxidase conjugated goat anti-rabbit antiserum (Cappel Laboratories) was used for detection of \textit{H. somnus} LPS specific antibodies in rabbit serums. The plates were incubated for 15 min, washed, and 100 ul of the substrate solution were added to each well. The substrate was prepared by adding 0.1 ml of 125 mM ABTS (2-2 Azino-di-(3-ethyl benzthiazaline-6-sulfonic acid) (Sigma) to 10 ml of 0.05 M citric acid pH 4 to which had been added 40 ul of a 1:500 dilution (v/v) of 30% hydrogen peroxide in 0.05 M citric acid. The plate was then
Dilute Antigen to Appropriate Concentration in Sodium Carb-Bi-carb Buffer pH 9.6

Add 100µl/Well (PVC Plates, Dynatech Labs) Incub. 37°C/3 Hr.

Wash 6X

Add 100µl 3% Normal Horse Serum in Wash Soln. to Each Well

Wash 6X

Add 100µl 3% Ovalbumin in Wash Soln. to Each Well

Incubate 20 min. 37°C/Covered Vibrating

Wash 6X

Add 50µl Peroxidase Conjugated Goat-anti-bovine Ig (KPI) at Appropriate Dilution to Each Well

Incubate 15 min. /37°C/Vibrating/Covered

Wash 6X

Add 100µl Substrate (0.1ml of 125mm ABTS/10ml 0.05 M Citric Acid pH 4.0 and 40µl 1:500, 30%H₂O₂ in Citric Acid/10ml)

Incubate 20 min/37°C/Vibrating/Covered

Stop Reaction by Adding 50µl 0.5% Hydrofluoric Acid to Each Well

Read Absorbance at 405nm

Figure 2. ELISA procedure
incubated for 20 min at 37 C, uncovered, with shaking, in the dark after which the reaction was stopped by adding 50 ul of 0.5% hydrofluoric acid in DW (v/v) to each well and incubating the plate as before for 5 min. The color reaction was stable for up to 2 h. The resulting absorbances were read, both visually and on a ELISA reader (Titertech Multiskan Microelisa Reader, Dynatech) at 405 nm. Serum antibody titers were determined by comparing the absorbance of the unknown serum dilutions with the absorbance of the known negative serums.

Clinical data

Clinical data consisted of noting clinical signs, rectal temperature, and post mortem examination of the animals that died.

Results

Detection of antibodies to H. somnus antigens in rabbit serums

The results of the rabbit immunoglobulin ELISA using various H. somnus antigens showed that partially purified LPS had the lowest cross-reactivity with other common bovine pathogen antiserums. The incubated extract antigen cross-reacted with A. lignieresii, B. bronchiseptica, L. monocytogenes, M. bovis, P. multocida, S. agalactiae, and P. hemolytica as did the lysozyme extract antigen. The heat-killed supernatant antigen cross-reacted with antiserums to
Figure 3. Comparison of absorbances obtained using the five *H. somnus* antigens to determine cross-reactivity with other common bovine pathogens.
M. bovis, P. multocida, S. agalactiae, P. hemolytica, A. lignieresii and B. bronchiseptica and with L. monocytogenes and C. pyogenes to a lesser degree. The LPS antigen was chosen as the most specific antigen for the ELISA test because, although it showed cross-reactivity with a number of the antiserums, the absorbance was nearly that of the negative serum or background absorbance.

Detection of antibodies to H. somnus LPS in bovine serums

The ELISA test for the detection of specific anti-H. somnus LPS immunoglobulins in bovine serum was conducted with all components in excess except the amount of antibody in the test serum. The LPS concentration of 0.1 ug/well was determined to be the optimum amount that gave little non-specific binding of conjugate, yet detected minimal amounts of antibody. Binding of the LPS antigen to the polyvinyl chloride solid phase in fresh 0.125 M sodium-carbonate bicarbonate buffer pH 9.6 by incubation for 3 h at 37 C and then overnight at 4 C gave reproducible results. Optimal conjugate concentrations for each lot of conjugate was determined by measuring conjugate binding to positive and negative serum samples in the ELISA test as a function of concentration. The results showed that a 1:1000 dilution in PBS, of the particular lot used, was in excess for detection of antibody but did not non-specifically bind to antigen. The optimum conjugate incubation time of 15 min was chosen because binding to anti-LPS antibody was accomplished without non-specific binding to the LPS antigen, as detected in control wells. Twenty min
was chosen as the incubation time for substrate giving optimum color change for positive serums. Time periods longer than 20 min gave non-specific color changes in wells with negative serums or very dilute positive serums.

Brown et al. (14) concluded that a significant CF titer was a four-fold increase of at least 70% complement fixation over the level of convalescent serum. Because of the low background with the ELISA assay reported here we believe significant titers are any absorbance of at least 0.1 nm over that of the negative control serums at the same dilution.

Titers to \textit{H. somnus} LPS in calf serum was determined during the peak of the \textit{H. somnus} infection season in Iowa, Nov.-Jan. (2). Serum from 63 control calves at 7 intervals in the three month period were tested. The titer, as determined by comparison of the absorbance at various dilutions to that of negative serum, showed a general upward trend for the entire herd. The response of individual animals varied. Some showed very little increase in titer, some had little or no titer, still others showed a marked increase in antibody to LPS. Several of the animals in the herd died from pneumonia and others showed obvious signs of respiratory disease. The antibody responses of those that became ill indicated that several of these animals had a marked decrease in serum antibody titer after illness. Two animals that became ill showed an increase in serum antibody during and after illness. All animals were treated with antibiotics at the first sign of illness. Of the three that died of respiratory disease, two had
Figure 4. Comparison of the humoral antibody response to *H. somnus* LPS in two separate calf herds that had been naturally infected with *H. somnus*.
only a marginal (1:20) titer that did not increase before death. The third showed a drop in serum antibody level just prior to death. Comparison of the anti-*H. somnus* LPS antibody response between two cattle herds was conducted. The average serum antibody titer for the young herd studied above was calculated for each day that the serums were collected. These values were compared with serum antibody levels from another herd of older calves that suffered a naturally occurring respiratory infection in the spring and recovered. The herd of young calves that became ill never developed the antibody levels that the older group did. During the disease outbreak, the mean antibody titer of animals in the young herd was less than 1:8 while the mean antibody titer of animals in the older herd during the disease outbreak was 1:160. By the time the older herd had recovered, they had an average serum antibody titer of greater than 1:640.

**Serum antibody response to vaccination**

Both groups of vaccinates, the Somnugen vaccinated (S) group and the ribosomal vaccinated (R) group, showed a significant increase in antibody titer over the control group after immunization. All three groups developed an increase in titer with the S group showing the greatest increase. None of these animals were experimentally challenged with live *H. somnus*; however, in the middle of the second week individuals in all three groups developed respiratory distress, ranging from labored breathing to pneumonia. All animals showing signs of illness were treated with antibiotics. Of those that became
Figure 5. Humoral response to *H. somnus* LPS following vaccination with either a commercial bacterin or an experimental ribosomal vaccine.
clinically ill four were in the S group, five were in the R group and one was in the C group. All ten animals recovered after antibiotic treatment.

Discussion

The presence of \textit{H. somnus} anti-LPS antibody in bovine serum can be measured by using an ELISA technique which employs phenol-water extracted LPS as the antigen. The specificity of the ELISA relies in part on the purity of the antigen prepared by the phenol-water extraction method and subsequent alcohol precipitation to remove nucleic acids and proteins from the preparation. With the results presented here the LPS antigen from \textit{H. somnus} strain 8025 appears to be specific for \textit{H. somnus} antibodies as indicated by the absence of cross-reactivity with antiserums to various bovine pathogens. In contrast a diethylene glycol extract, lysozyme extract, heat extract and an incubation extract showed cross-reactions with a number of the antiserums against common bovine pathogens. These pathogens include \textit{A. lignieresii}, \textit{B. bronchiseptica}, \textit{C. fetus}, \textit{L. monocytogenes}, \textit{M. bovis}, \textit{P. multocida}, \textit{P. haemolytica}, \textit{S. agalactiae}, and \textit{C. pyogenes}, which is consistent with results of other workers which have reported common antigens associated with \textit{H. somnus} and other bovine pathogens (37,48,73,91,123,136). However, contradictory reports of cross-reactions of \textit{H. somnus} antigens with other organisms have been reported.
Canto et al. (21) reported that cross-reactivity of *H. somnus* antibodies depended greatly on the type of serological procedure and method of antigen preparation employed. They noted the greatest cross-reactivity in agglutination tests using whole cell antigens. They developed an ELISA test using a supernatant of a saline extract produced by incubating a standardized number of unwashed cells in PBS at 4°C for 24 h, as their ELISA antigen which they concluded consisted largely of an *H. somnus* common antigen and which showed cross-reactivity on the ELISA test with antigens from *H. agni* only. This saline extracted antigen would be similar to the incubation extract antigen described in this paper which cross-reacted with *P. multocida* antisera in our ELISA test.

Partially purified LPS showed the least cross-reactivity of any antigen we tested, but had good reactivity with anti-*H. somnus* serums and was chosen as the specific antigen for the ELISA test overcoming the common problem of cross-reactivity associated with other serological methods. This ELISA test, using *H. somnus* LPS as the antigen, showed high sensitivity with positive titers in hyperimmune rabbit serum to 1:10,240 and to 1:5120 in immunized bovine serum, and satisfactory precision between replicate measurements of the same and different serum samples from day to day and from worker to worker. All serums from animals in group A, injected with one of the two *H. somnus* vaccines showed a five-fold increase in antibody titer while control non-vaccinates of the same herd showed a 2.5-fold increase in antibody titer during the one month testing period. During this time,
the animals became ill with respiratory disease of undetermined etiology, but probably due to *H. somnus*, which may account for the rise in antibody levels in the control group. The fact that animals in all three groups became clinically ill may indicate that humoral immunity alone is not sufficient to completely protect the animal from infection by *H. somnus*.

Group B, consisting of 63 cattle, was followed for two months during which serum samples were collected for evaluation of serum antibody to *H. somnus* LPS. During the time of testing, this herd suffered an attack of respiratory disease. *H. somnus* was recovered from the animals that died. The herd showed an overall increase in anti-*H. somnus* LPS antibody level up to a mean titer of 1:160, although individual antibody levels varied greatly. Of particular interest were the serum antibody levels of those animals that became severely ill but survived and those that died. Several of the sick animals showed a decrease in antibody levels during illness, two however showed a moderate increase in antibody levels. Of those that died, two had a low (1:20) level of antibody to *H. somnus* LPS and the third animal had a decrease in antibody titer prior to death. Animals that died were confirmed to have had pneumonia caused by *H. somnus*.

In another herd of naturally infected cattle, that had been clinically infected by *H. somnus* and had recovered, a much higher average serum antibody level to *H. somnus* LPS (1:640) was found. The importance of these findings is speculative but indicates a role for humoral immunity in recovery from *H. somnus* infections. Antibody specific for
*H. somnus* LPS may help neutralize the pathogenic activities of the LPS antigen which may occur during infection; however, these results indicate that vaccination does not provide sufficient protection against disease caused by *H. somnus*.

Antibody against LPS can function as an antitoxin and as such can diminish the mortality from gram-negative bacteremias (13). It has been reported that anti-LPS antibody can neutralize the effects of meningococcal endotoxins (31). High titers of antibody to core LPS at the onset of human bacteremia has been associated with significantly less frequent shock and death in patients with bacteremia due to gram-negative bacilli (160).

To reach any final conclusions about the clinical significance of the results obtained by this ELISA assay will require more extensive and controlled studies. This assay will be employed for an ongoing evaluation of the humoral response to *H. somnus* infections and vaccination to assess whether the rate and amount of antibody increase correlates with certain respiratory disease syndromes and recovery from disease. If possible, we also hope to evaluate the feasibility of using this ELISA assay as an aid in the diagnosis of *H. somnus* infection in cattle and the assessment of response to therapy instead of the commonly applied complement fixation test (CFT) because of the increased specificity of the ELISA test over the CFT.
SECTION II.

EFFECT OF HAEMOPHILUS SOMNUS FRACTIONS
ON BOVINE POLYMORPHONUCLEAR LEUKOCYTE FUNCTIONS

Summary

The effect of Haemophilus somnus on bovine polymorphonuclear leukocyte (PMN) function was examined in vitro with whole cells and fractions extracted from the surface of this bacterium. The ability of PMNs to iodinate protein and ingest Staphylococcus aureus was significantly inhibited in the presence of live cells, heat-killed whole cells or supernatant fluid from heat-killed cells, but not in the presence of washed, heat-killed cells. None of the fractions inhibited nitroblue tetrazolium (NBT) reduction by PMNs. The PMN inhibitory factors were further characterized. The material that inhibited S. aureus ingestion was found to be a heat-stable cell surface material of greater than 300,000 MW. The fraction inhibiting iodination of protein was found to be less than 10,000 MW.

Introduction

Haemophilus somnus (H. somnus) is recognized as a cause of several disease syndromes of cattle. H. somnus was first recognized
as the bacterium that caused infectious thromboembolic meningoen cephalitis (TEME) in cattle (7,14,73,81,101,104,144). The bacterium has since been discovered to be a major etiological factor in a variety of bovine disease syndromes, including respiratory infection, septicemia, and resulting thrombosis (14). Therefore, \textit{H. somnus} is considered an important pathogen which causes great economic loss to the cattle industry (118).

Lesions produced in cattle by infection with \textit{H. somnus} are characterized by the presence of large numbers of polymorphonuclear leukocytes (PMNs). Cattle experimentally infected with \textit{H. somnus} exhibit a neutrophilic leukocytosis within 8 hours of challenge (153). Microscopically, vasculitis and neutrophilic cuffing has been observed in the brain along with neutrophil infiltration of the submucosa of the trachea (37). Vasculitis, thrombosis, microabscessation and focal necrosis were also observed in spinal cord, kidney, bladder, mucosa of the gastrointestinal tract, myocardium, skeletal muscles and retina (138). These areas of thrombosis and vasculitis are often infiltrated by neutrophils (73,104,138).

PMNs are one of the major cellular defense mechanisms in protecting animals from microbial infection. The nature of the interaction of the bacterium with the PMN may determine whether or not infection occurs. The nature of the infectious process suggests that \textit{H. somnus} may escape host defense mechanisms. Therefore the in vitro interaction of \textit{H. somnus} cells and fractions derived from those cells with PMNs were evaluated. Major suppressive effects were observed on
PMN functional activities. It has been postulated that the occurrence of bacteremia in the face of a high serum antibody titer indicates that the host phagocytic system is no longer functioning effectively. Evidence for this hypothesis has been reported. Only 55% of the organisms of either a pathogenic or non-pathogenic strain of H. somnus were phagocytized in vitro, while antiserum to the pathogenic strain decreased the percentage of the bacteria phagocytized (126). In most infectious diseases pathogenicity is related to more than one microbial characteristic (93). A correlation can often be made between cell surface factors and pathogenicity. If these factors that increase the pathogenicity of an organism were identified it could be important in understanding the mechanism by which the microorganism invades a host and the possible prophylactic measures that can be used against the pathogen.

Materials and Methods

Bacterial propagation

H. somnus strain 8025 was used throughout these experiments. This strain was initially isolated at the Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa, from a bovine brain with lesions of infectious thromboembolic meningoencephalomyelitis. The bacterium, which had been stored at -70 C in egg yolk culture (2 ml), was inoculated into 10 ml of brain heat infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% normal bovine serum
and 5% yeast extract (Difco) (BHISY). This broth culture was incubated for 12 h at 37 °C in an atmosphere of 5% CO₂ and 95% air and then used to inoculate Roux flasks containing the BHISY medium and 1.5% Bactoagar (Difco). The bottles were rotated by hand to spread the inoculum over the agar surface and the bottles were incubated for 48 h. The bacteria were harvested by adding 5 ml of 0.015 M phosphate buffered saline solution (pH 7.2, PBS) to each bottle, scraping the agar surface with a sterile wire and aspirating the suspended bacteria from the bottle. The harvested cells were pooled and washed 3 times in PBS by alternate centrifugation at 10,000 x g for 30 min at 5 °C and resuspension. After the last wash, the bacteria were resuspended in PBS to a 10x concentration of a suspension with an optical density (O.D.) of 0.4 at 600 nm which was equivalent to 4.2 x 10⁸ cells/ml.

**Bacterial fractions**

Bacteria suspended in PBS were incubated in a water bath at 60 °C for 1 h. After this heat extraction an aliquot was saved for the "heat-killed cells and supernatant" (HKC+S). The remainder was centrifuged at 10,000 x g for 20 min at 5 °C. The pellet was resuspended in PBS to the original volume ("heat-killed cells" or HKC). The supernatant fluid was termed heat extract (HE).

An aliquot of HE was saved and the remainder was filtered through a 300,000 molecular weight cutoff filter (Amicon Corporation, Lexington, MA). The retentate was resuspended to the original volume in PBS and considered the heat extract fraction of greater than
Figure 1. Preparation of *H. somnus* fractions
300,000 MW (HE300R). The filtrate was divided into two aliquots, one of which was saved and called the heat extract of less than 300,000 MW (HE300F). The other aliquot was passed through a 10,000 MW cutoff filter (Amicon) and called the heat extract of less than 10,000 MW (HE10F).

**PMN preparation**

PMNs were prepared by the method of Roth and Kaeberle (1980). Peripheral blood was collected from apparently healthy cattle into ACD solution. After the blood was centrifuged at 1000 x g for 20 min, the plasma and buffy coat layers were aspirated and discarded. The RBCs were lysed by the addition of 2 volumes of cold phosphate buffered pH 7.4 deionized water for 50 seconds at which time the isotonicity was restored by adding 1 volume of cold phosphate buffered pH 7.4, 2.7% NaCl.

The PMNs present in the RBC lysate were pelleted by centrifugation and washed once with PBS. The cell suspension was adjusted to a final concentration of 50 x 10^6 PMNs per ml in PBS and used in all three PMN function tests. Differential stains indicated that these preparations contained an average of 96% neutrophils and 3-4% eosinophils.

**PMN function tests**

The procedures for evaluating PMN function have been described in detail previously (115). All PMN function tests were conducted in
duplicate and the average of duplicate values were used for calculations.

**Iodination** The iodination test was conducted in 12 x 75 mm polyethylene snap cap tubes (#2050, Falcon, Oxnard, Ca.). The standard reaction mixture contained 2.5 x 10^6 PMNs, 0.05 mCi of 125 Iodine (Carrier free in 0.1 M NaOH, New England Nuclear, Boston MA), 40 nmole of NaI, 0.05 ml opsonized zymosan (10 mg/ml) and 0.3 ml Earles balanced salt solution (EBSS, GIBCO, Grand Island, N.Y.). To determine the effect of bacterial fractions on iodination by PMNs, 0.05 ml of *H. somnus* fractions (4.8 x 10^6 cells or equivalent soln.) or 0.05 ml of PBS for controls were added to the standard reaction mixture. The reaction was allowed to proceed for 20 min at 37 C as the tubes were tumbled end over end. The amount of trichloroacetic acid (TCA)-precipitable radioactivity was determined. The results are expressed as a percent of control values and plotted as bar graphs.

**Nitroblue tetrazolium (NBT) reduction** The NBT test was conducted in duplicate in 16 x 100 mm siliconized glass tubes. The standard reaction mixture contained 0.2 ml of NBT solution (2 mg/ml), 5 x 10^6 PMNs, 0.1 ml preopsonized zymosan preparation (10 mg/ml), 0.1 ml PBS in control tubes or 0.1 ml of the bacterial fractions diluted in PBS and sufficient EBSS to bring the total volume of the reaction mixture to 1.0 ml. All of the reactants except the PMNs were added to the tubes and allowed to equilibrate in a 37 C waterbath. The tubes were left in the waterbath and the reaction was initiated by adding the PMN suspension to each tube. The tubes were incubated in the
shaking water bath for 10 min at 37 C after which the reaction was stopped by adding 5 ml of cold 1 mM n-ethylmaleimide diluted in saline. The cells and the precipitated formazan were pelleted by centrifugation at 500 x g for 10 min, the supernatant fluid was discarded and the pellet was resuspended in 5.0 ml of pyridine. Formazan was extracted by brief sonification of each tube followed by heating in a boiling waterbath for 10 min. The pyridine-formazan was clarified by centrifugation at 500 x g for 10 min and the O.D. was immediately determined at 580 nm in a spectrophotometer using a pyridine blank.

**Staphylococcus aureus ingestion**

Heat-killed (125I) iododeoxyuridine ([125I] UdR, New England Nuclear, Boston, MA)-labeled *S. aureus* was used to evaluate ingestion by PMNs. The test was conducted in 12 x 75 mm plastic tubes and the standard reaction mixture contained 0.1 ml of ([125I] UdR labeled) *S. aureus*, 0.05 ml of PMNs (25 x 10^6), (bacteria to PMN ratio = 60:1), 0.05 ml of a 1:10 dilution of bovine anti-*S. aureus* serum and 0.3 ml of EBSS. To determine the effect of bacterial fractions on ingestion by PMNs, 0.05 ml of *H. somnus* fraction (6 x 10^6 cells or equivalent soln.) or 0.05 ml of PBS as a control were added to the standard reaction mixture. The reaction was allowed to proceed for 10 min at 37 C in a shaker waterbath after which the extra-cellular, non-phagocytized *S. aureus* was removed by adding Lysostaphin (Sigma Chemical Co., St. Louis, Mo.) to the reaction mixture followed by incubation for an additional 20 min. The tubes were then filled with PBS and the PMNs were washed
twice by alternate centrifugation and resuspension. The amount of PMN-associated radioactivity was determined and the results were expressed as percentages of (125I) UdR-labeled _S. aureus_ that was ingested. The values for the tubes containing bacterial fractions were then calculated as a percent of the control values.

**Data analysis** To determine the effect of bacterial fractions on each PMN function the value obtained when a bacterial fraction was added to the PMNs was compared to the value obtained in control reactions (with PBS only). An analysis of variance procedure was used to determine significance of the differences in PMN function due to addition of the bacterial fractions. For the graphic presentation of the data, all treatment values were expressed as a percentage of the untreated controls.

**Results**

**Effect of H. somnus fractions on iodination** The ability of PMNs to iodinate protein in the presence of _H. somnus_ fractions was determined to study the effect of these fractions on the myeloperoxidase-hydrogen peroxide-halide antibacterial system of the PMN. The value for iodination by PMNs in control reactions was 37.99 ± 12.02 (mean ± S.E.M.) (n = 16) nmole NaI per 10^7 PMNs per hour. The ability of PMNs to iodinate proteins in the presence of live cells, HKC+S, HE and HKC was determined. HKC+S and HE suppressed the iodination reaction. HKC had no effect on iodination by PMNs. The HE was processed for further characterization of the inhibitory factor by
filtration through two filters, one with MW cutoff of approximately 300,000 (300K) and the other with MW cutoff of approximately 10,000 (10K). Both HE and the HE filtrate from the 10K filter (HE10F) inhibited iodination by 25%. The He retentate from the 300K filter (HE300R) did inhibit iodination but only by 9%.

**Effect of *H. somnus* fractions on *S. aureus* ingestion**

To determine the effect of *H. somnus* fractions on phagocytic activity, PMNs were added to a standard suspension of opsonized *S. aureus* in the presence or absence of *H. somnus* fractions. The PMNs in control reactions ingested 26.01 ± 11.75 (mean ± S.E.M.) (n = 13) percent of the *S. aureus* in the reaction mixture. *S. aureus* ingestion was inhibited in the presence of live cells, HKC+S, HKC and HE. To further characterize the inhibitory factor, HE was filtered through the two filters as described previously. HE300R inhibited *S. aureus* ingestion by 50%. Neither HE300F or HE10F had an effect on *S. aureus* ingestion by PMNs.

**Effect of *H. somnus* fractions on NBT reduction**

To study the effect of *H. somnus* fractions on the oxidative metabolism of PMNs, the ability of PMNs to reduce NBT by the production of superoxide anion in the presence of the bacterial fractions was determined. The value for NBT reduction by PMNs in control reactions was 0.499 ± 0.144 (Mean ± SEF) (n = 18). None of the *H. somnus* fractions had a significant effect on NBT reduction (p < 0.05).
Figure 2. Effect of *H. somnus* fractions on iodination by bovine PMNs stimulated with opsonized zymosan. Values represent mean percentages (+- standard deviation) of the control value. Statistically significant differences from control values are indicated as **, p < 0.05; n=16
Figure 3. Effect of *H. somnus* fractions on *S. aureus* ingestion by bovine PMNs. Values represent mean percentages (+ SD) of the control value. Statistically significant differences from control values are indicated as **, p < 0.05; n=13
Figure 4. Effect of *H. somnus* fractions on NBT reduction by bovine PMNs stimulated with opsonized zymosan. Values represent mean percentages (± standard deviation) of the control value. None of the differences were statistically significant, $p < 0.05$; $n=18$
Discussion

Phagocytic cells occupy a central position in host defense against infection by microorganisms and neutrophils are primarily responsible for the host defense against obligate extracellular pathogens. A major function of the PMN is the phagocytosis and destruction of these invading microorganisms. Some microbial pathogens have survival properties to protect against the killing mechanisms of phagocytic cells. These properties increase the pathogenicity of the organisms. These anti-phagocytic mechanisms include avoidance of recognition, inhibition of chemotaxis, attachment, ingestion, oxidative metabolism or degranulation, and possibly the elaboration of a leukocidic factor (113). Any of these inhibitory actions on PMN function should be considered as virulence factors of the pathogenic microorganism (34).

The ingestion of bacteria is the first step in the bactericidal activity of the PMN. The results of the S. aureus ingestion assay indicate that cell components of H. somnus inhibit the ability of PMNs to ingest particles. When bacterial fractions containing whole organisms were used, the inhibition of ingestion of S. aureus may have been due to competition between the two bacterial species. Since soluble bacterial fractions would not be phagocytized, they should not compete with particulate materials in the S. aureus ingestion assay. The factor associated with H. somnus which inhibited S. aureus ingestion is soluble and greater than 300,000 MW. This factor may be
heterogeneous in composition and may be comparable to a fraction extracted by Stephens et al. (135). These researchers isolated two antigens from the outer membrane complex of *H. somnus* using a saline extraction of whole *H. somnus* cells for use as possible vaccines, and showed these fractions to be greater than 150,000 MW.

Oxidative metabolism of the PMN is an important aspect of its bactericidal activity (5,113). When a PMN receives the proper stimulus, an oxidase enzyme on the surface of the plasma membrane or phagosomal membrane will catalyze the conversion of oxygen to superoxide anion. This reaction is known as the respiratory burst. Superoxide anion spontaneously dismutates to hydrogen peroxide. NBT is directly reduced by the superoxide anion to an insoluble purple formazan (158). NBT reduction is therefore a measure of superoxide anion generation by the PMN. Since NBT reduction was not inhibited by whole bacteria or bacterial fractions, *H. somnus* apparently does not inhibit the production of superoxide anion by PMNs.

Associated with the PMN oxidative metabolism is the iodination reaction. This reaction is a measure of the ability of PMNs to convert inorganic iodide to a trichloroacetic acid-precipitable (protein-bound) form and occurs inside the phagocytic vacuole via the action of hydrogen peroxide plus myeloperoxidase from the primary granules in the PMN. This reaction is dependent upon the generation of hydrogen peroxide during the respiratory burst, degranulation to release myeloperoxidase, the presence of iodine, the unimpaired ability of myeloperoxidase to catalyze the reaction, and the presence
of tyrosine to bind iodine. This system has been reported to kill bacteria, fungi and viruses (9,125). Hydrogen peroxide is formed from superoxide anion. NBT reduction was not inhibited by the *H. somnus* fractions indicating that the formation of superoxide anion and presumably hydrogen peroxide was not impaired. It is possible that the bacterial fractions inhibited degranulation of the primary granule into the phagosome or that myeloperoxidase activity was inhibited.

Little is known about the mechanisms that allow *H. somnus* to survive in the bovine respiratory tract or to develop a septicemia. *H. somnus* is commonly isolated from healthy cattle without causing signs of disease. During conditions of stress, the bovine host apparently becomes more susceptible to infection by *H. somnus*. In vitro studies have shown that *H. somnus* can be opsonized and killed by bactericidal antibodies from vaccinated cattle in conjunction with complement and neutrophils. It does not appear that all *H. somnus* organisms are normally killed however (126). The neutrophil suppressive factors associated with *H. somnus*, reported in this paper, may act in conjunction with other immunosuppressive events to hinder the host defense mechanisms enough for an *H. somnus* infection to become established. Immunosuppressive effects that could help establish an *H. somnus* infection may include increased corticosteroid levels associated with stress. High plasma cortisol has been reported to inhibit both the cellular and humoral immune response. Also, high serum cortisol levels inhibit the hydrogen peroxide-halide-myeloperoxidase bacterial killing system (114). The combined effects
of stress and *H. somnus* factors may suppress neutrophil function to 
the point that infection leads to a disease process.

Some bacterial pathogens circumvent recognition as foreign by the 
host surveillance mechanisms (51) or inhibit chemotaxis of neutrophils 
(34,64). It does not appear that inhibition of recognition or 
impairment of chemotaxis of neutrophils occurs in *H. somnus* 
infections. An abundance of neutrophils at the site of infection is a 
common pathologic sign in *H. somnus* disease syndromes, indicating 
intact and even extreme chemotaxis of neutrophils to the site of 
infection. In cases of abortion, where *H. somnus* is implicated, 
abundant neutrophils are found in fetal airways (2,25,144). 
Neutrophil infiltration of synovia is noted in cases of weak calf 
syndrome when *H. somnus* is an expected cause (147). In cases of 
pneumonia, Andrews et al. (2) described microscopic lesions of 
peribronchiolar filling of alveoli with albuminous fluid and 
neutrophils. Gross lesions associated with TME caused by *H. somnus* 
occur in the brain and irregularly in the spinal cord, mucosa of the 
esophagus, gastrointestinal tract and urinary bladder. Histological 
examination reveals severe vasculitis, thrombosis, necrosis of vessel 
walls and intense neutrophil accumulation associated with the lesions 
(73,104,138). Many bacterial pathogens produce a capsule that enables 
the microbe to resist attachment to and ingestion by host phagocytes. 
*Y. pestis* is reported to acquire a capsule only after infecting a host 
(154). *H. somnus* capsulization has been reported by Miller et al. 
(91) and Williams et al. (153) but refuted by Stephens and Little
(137) who examined the cell with electron microscopy. Stephens and Little have shown that the cell envelope is composed of an outer membrane, peptidoglycan layer and an inner cytoplasmic membrane. Also, EM studies of *H. somnus* adherent to endothelial cells in tissue culture did not demonstrate pili (143). It remains possible that certain strains of *H. somnus* may possess a capsule or may acquire a capsule only in the host, enabling *H. somnus* to resist phagocytosis. The *H. somnus* strain used in this study, strain 8025, was not examined for capsule formation.

Viral induced suppression of neutrophil function may play a role in the pathogenesis of secondary bacterial infections (43). *H. somnus* fractions did not inhibit the oxidative metabolism of bovine neutrophils phagocytizing particles of opsonized zymosan. However, primary or simultaneous viral infection and subsequent depression of neutrophil oxidative metabolism may be involved in the pathogenesis of *H. somnus* infections. Pennell and Renshaw (106) speculated that primary viral infection inhibiting bactericidal activity in the lung may be a predisposing factor in *H. somnus* caused disease syndromes.

Resistance to microbicidal oxygen products can be achieved by a number of bacterial mechanisms. Catalase-rich strains of staphylococci had a better survival rate and decreased myeloperoxidase dependent iodination by neutrophils, probably due to destruction of hydrogen peroxide (34). Although there have been some reports of catalase positive isolates, *H. somnus* is considered catalase negative and hence would not destroy hydrogen peroxide. The oxidative
potential of singlet oxygen is quenched when in contact with substances with a large number of conjugated double bonds, e.g. carotenoids (34). *H. somnus* characteristically has a bright yellow color. This pigmentation varies between *H. somnus* strains but may serve to quench singlet oxygen produced during the oxidative burst (26). It is apparent that the virulence of a microorganism is associated with its ability to interfere with the normal immune response of the host. *H. somnus* apparently has one or several virulence factors associated with it. These factors enable this organism to produce the disease syndromes known as the "*H. somnus* complex". The research reported in this paper has shown that soluble surface bacterial fractions from *H. somnus*, isolated by heating the organisms in saline, inhibit PMN ingestion of opsonized *S. aureus* and iodination of protein. The mechanisms of these two effects are still under investigation. These fractions may be virulence factors acting to induce the disease state. Whether specific antibody to these factors will neutralize their effect and reduce virulence remains to be seen.
The experimentation reported in this thesis includes two subjects: (1) development of a specific and sensitive ELISA test to evaluate the humoral immune response of cattle to *H. somnus* LPS during infections and vaccination, (2) demonstration of *H. somnus* virulence factors that suppress two neutrophil functions.

Five antigen preparations were isolated from *H. somnus*. These five antigens were used in the ELISA assay. Rabbit antiseraums to ten common bovine pathogens were made and used to determine the specificity of the five *H. somnus* antigens for use in the ELISA procedure. It was found that phenol-water extracted LPS of *H. somnus* was the most specific antigen. This antigen was used to monitor anti-*H. somnus* LPS antibody levels in a herd of calves during the months of highest incidence of *H. somnus* infections. During the test period, a number of the calves developed respiratory disease. *H. somnus* was isolated from those animals that died from pneumonia. Variable responses to *H. somnus* LPS were seen among individual animals. Of the animals that became clinically ill, several showed a decrease in serum antibody titer after illness, two had an increase in titer after illness. All animals were treated with antibiotics at the first sign of illness. Of the three animals that died, two had low titers at the time of death while the third showed a drop in antibody level prior to death. Animals immunized with one of two vaccines developed an increase in antibody titers over a control group. The ELISA test
presented here is specific and sensitive. It can be used for further studies on the humoral immune response of cattle to *H. somnus* and possibly as a diagnostic aid.

The role of the humoral immune response in protection from infection and recovery from infection by *H. somnus* in cattle remains a mystery. Serological surveys have shown that almost all cattle have antibody to *H. somnus* (14, 82), but in vitro studies suggest that animals succumbing to *H. somnus* infection may lack normal humoral defense factors (127). The most susceptible age group, with the highest incidence of *H. somnus* infection, were young calves between five months to one year of age. This group had lower bactericidal activity than adults and it appears that the anti-bacterial antibodies were not maternal in origin but eventually appear in serum due to increased exposure to *H. somnus* or due to maturation of the calves' immune system. Simonsen (126) has reported that in vitro serum bactericidal activity varies with the age of the animal, and is complement dependent with the concentration of anti-*H. somnus* antibody being the limiting factor. Pennell and Renshaw (106) also reported substantial levels of bactericidal activity with a combination of complement, leukocytes and specific opsonin, anti-*H. somnus* antibody.

Stephens (134) reported that serum titers do not determine susceptibility to challenge or the outcome of infection with *H. somnus*. They found increasing titers in cattle 2-3 days post challenge indicating an anamestic response in cattle acutely ill with TE ME. They proposed that high antibody titers in cattle with
septicemia may contribute to the pathogenesis of disease. They also reported evidence that indicates the host's phagocytic systems are not functioning effectively and that antigen-antibody complexes are probably not phagocytized. Stephens (134) also found that calves without antibody did develop septicemia but not TEME after IV inoculation of live H. somnus. The results reported in this thesis support the idea that antibody contributes to the severity of disease in some cases. However, the low titers to H. somnus LPS obtained during immunization and natural infection indicate that H. somnus is not highly immunogenic. The limited humoral response to H. somnus may therefore also contribute to the establishment of disease.

These results reflect our present understanding of the role of humoral immunity in H. somnus disease which is conflicting and complicated. This confusion probably reflects the complexity of the overall immune response to H. somnus infection which is often preceded by stress, viral infection, cold weather or other factors which may each elicit a different effect on the host immune system.

The neutrophil function results indicate the heat killing of H. somnus releases at least two virulence factors into the medium. Two separate neutrophil functions were suppressed by the bacterial fractions of differing molecular weight. One fraction, of less that 10,000 MW was found to inhibit iodination of protein, an important step in the potent myeloperoxidase - halide - hydrogen peroxide killing mechanism of neutrophils. The second fraction, of greater than 300,000 MW was found to inhibit the ingestion of radiolabelled S.
The inhibition of these two neutrophil functions corresponds with the pathologic findings of *H. somnus* infections which occur in the presence of an accumulation of neutrophils. Virulence factors of *H. somnus* had been suspected but not previously demonstrated. These two fractions from *H. somnus* can be considered as probable virulence factors that are involved in the pathogenesis of *H. somnus* infections.

The overall picture of *H. somnus* disease presents a combination of bacterial and host responses and it is this joint action that is probably responsible for the disease syndromes and lesions found in *H. somnus* infections. It is probable that *H. somnus*, properly opsonized with both antibody and complement, are phagocytized and killed by neutrophils. However, it is possible that not all of the *H. somnus* organisms are phagocytized. EM studies of neutrophils incubated with opsonized *H. somnus* should be undertaken to resolve whether the bacteria are ingested, are enclosed in a proper phagolysosome and survive ingestion. Two other aspects that should be investigated are whether *H. somnus* can produce polyanionic degranulation inhibitors or whether *H. somnus* can effect the cAMP levels in the neutrophil which are two mechanisms that enable some mycobacteria to resist phagocytosis (18,34).

I would like to present a possible pathogenic mechanism for *H. somnus* to stimulate further work in order to understand and control this important pathogen. First, *H. somnus* is common in the environment of its host, cattle. The bacterium has been commonly isolated from the tracheas of normal cattle (29), normal reproductive
tracts of cattle, and in high numbers from the prepuce of bulls (26). These sites could serve to easily spread the organism by inhalation, by ingestion, venereally or in the urine. Normally, the organism is controlled by host resistance mechanisms such as the mucociliary blanket of the trachea, neutrophils, etc. However, during stress from any number of causes, including viral infection, extreme cold temperatures, handling, weaning, castration, vaccination or transportation, suppression of the normal immune functions may occur in the animal giving *H. somnus* a chance to infect the host. After infection the first line of defense, the neutrophils along with circulating antibody to *H. somnus*, attempts to control the spread of the organism. The presence of large numbers of neutrophils in lesions associated with *H. somnus* suggests neutrophil chemotaxis is working well, which is probably associated with complement fixation to opsonized bacteria and release of C3a and C5a, potent chemotactic attractants. The cell wall of *H. somnus*, which may or may not have a capsular layer in vivo, in either case contains virulence factors that suppress bacterial ingestion and killing by the phagocyte. The cell wall also contains LPS which may activate the complement pathway, degrading complement components and interfering with the fixation of complement components to the bacterial cell surface further hindering phagocytosis. Soluble antigens from the bacterial surface or the bacterial cell itself may bind with circulating antibody resulting in a population of immune complexes.

LPS, C5a, and immune complexes can all induce toxic effects on
endothelial cells, exposing the basement membrane which may lead to typical lesions associated with \textit{H. somnus} infections (18). These lesions include disseminated intravascular coagulation, vasculitis, thrombus formation, hemorrhagic necrosis, fibrinopurulent polyarthritis, synovitis, necrotic laryngitis, neutrophil accumulation within the thrombus, and glomerulonephritis (66). Kidneys and joints are also target organs of immune complex disease (18). Antibody thus may contribute to tissue damage. This scenario incorporates the classical signs associated with \textit{H. somnus} infections. Whether it is correct remains to be seen. It is hoped that this research and future work will lead to protective control measures against this pathogen.
LITERATURE CITED


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