Immunobiological properties of

Haemophilus somnus

by

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department:  Veterinary Microbiology and Preventive Medicine

Major:  Veterinary Microbiology and Preventive Medicine

Iowa State University
Ames, Iowa

1984

1486584

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GENERAL 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." 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.

LITERATURE REVIEW


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 world wide but is most prevalent in temperant 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

(73,119). 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 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 H. somnus without loss of virulence (67).

H. somnus has also been isolated from the female reproductive
tract (26,90,147). 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 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 H. somnus in the urogenital tract of cattle suggests
this site as an effective reservoir from which spread of H. somnus by
ingestion, inhalation or venereally may occur.


Characteristics of Haemophilus somnus


H. somnus bacteria are small, pleomorphic coccobacilli. They
occur in both short chains and filamentous forms (27,48,73).
Pleomorphism is reduced by in vitro passage (73,104). H. somnus is
non-motile, non-sporeforming and non-piliated (6). Recent reports
(20,26) suggest 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 pappillate 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 H. somnus for the presence of a capsule in vivo has not

been done. The fungi Cryptococcus neoformans and the bacterium

Yersinia pestis have been shown to acquire capsules only after they

have infected a host (34). Perhaps H. somnus behaves in a similar

manner.

## Pigmentation

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 biochemcal reactions of 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). H.

somnus produces cytochrome oxidase, reduces nitrates (6,48), acidifies

litmus milk (48), produces indole, produces hydrogen sulfide, and

ferments glucose (6,48). H. somnus is negative for gelatin

liquifaction (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. agni (73,91,123), Actinobacillus lignieresii (48,91),

Listeria monocytogenes (91), Campylobacter fetus (91), Streptococcus

agalactiae (91), Brucella abortus (123), Bordetella bronchiseptica

(123), Yersinia entercolitica 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, Neiserria 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,

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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 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 H. somnus positive (37,60,73,101,108). Cattle with low titers were considered susceptible to intraveneous 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 Haemophilus somnus

The host response to 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 phagocytized 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 TEME 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-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 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), Neiserria (15,50), Salmonella (22,128), Staphyloccus (155),

Streptococcus (116), Treponema (145), Vibrio (61), Yersinia (23),

Mycoplasma (62), Rickettsiae (56), Rhodococcus (41) and 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. gonorhoeae 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 myeloperoxdase 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 C1 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 N. gonorrhoeae
(58), N. meningitidis (112), K. pneumonia (130), S. aureus (88), B.
anthracis (74), C. fetus (87), Bacteriodes fragilis (102), P.
multocida (83), E. coli (64), S. pneumonia and S. pyogenes (45), H.
influenzae group B (141) and 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 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, 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 I.


DETECTION OF ANTIBODY TO HAEMOPHILUS 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 x 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 x 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 x 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 Shwartzman 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

Live Washed
H. somnus Cells

Extract with
10 parts W/W
Diethylene Glycol

Cold Phenol
Extraction

Standardize to
10x .4 O.D. (600nm)
in PBS

Incub. 37°C /18Hr.
5% $CO_2$

Incub. 37°C/18Hr.
in Lysozyme
Buffer

Crude Lipopoly-
Saccharide

Incub. 60C/1Hr.

Shake Mechanically
3 Days/Rm Temp.

Acetone Precip.

Centrifuge
3000xg/20 Min.

Centrifuge
3000xg/20 Min.

Centrifuge
3000xg/20 Min.

Dialysis

25% Ethanol Precip.

Pellet
Discard

Supernatant
Heat Killed
Supernatant
Heat Extract (HE)

Supernatant

Supernatant

Diethylene Glycol
Extract (DEG)

8 Vol. Ethanol
Precip.

Incubated
Extract (IE)

Lysozyme
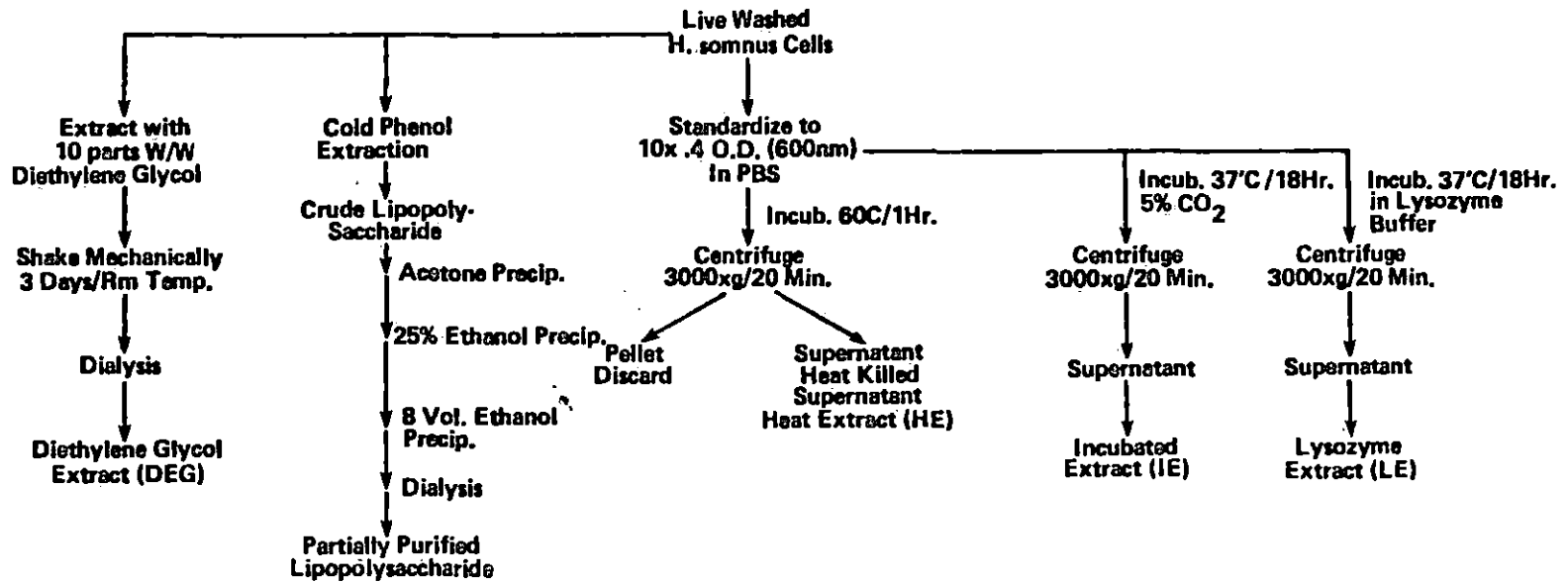Extract (LE)

Dialysis

Partially Purified
Lipopolysaccharide

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 anexperimental 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 5x10 -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 antiserums 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-H. somnus LPS antibodies in bovine serum while peroxidase conjugated goat anti-rabbit antiserum (Cappel Laboratories) was used for detection of 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

50 µl Antiserum

Mix w/50µl 0.05
Dithiothreitol in Tube

Incubate 37°C/30 Min.

Add 0.4ml Ovalbumin
in Wash Soln. to Tube to Give a
Final Serum Dil. of 1:10

Add 100µl of 1:10 Serum
to second Well in Each Row
and Dilute to 1:640
Transferring 0.1ml

Incubate 20 min.
37°C/Covered Vibrating

Wash 6X

Add 50ul 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 40ul 1:500,
30%$H_2O_2$ in Citric Acid/10ml)

Incubate 20 min/37°C/Vibrating/Covered

Stop Reaction by Adding 50µl 0.5%
Hydrofluoric Acid to Each Well
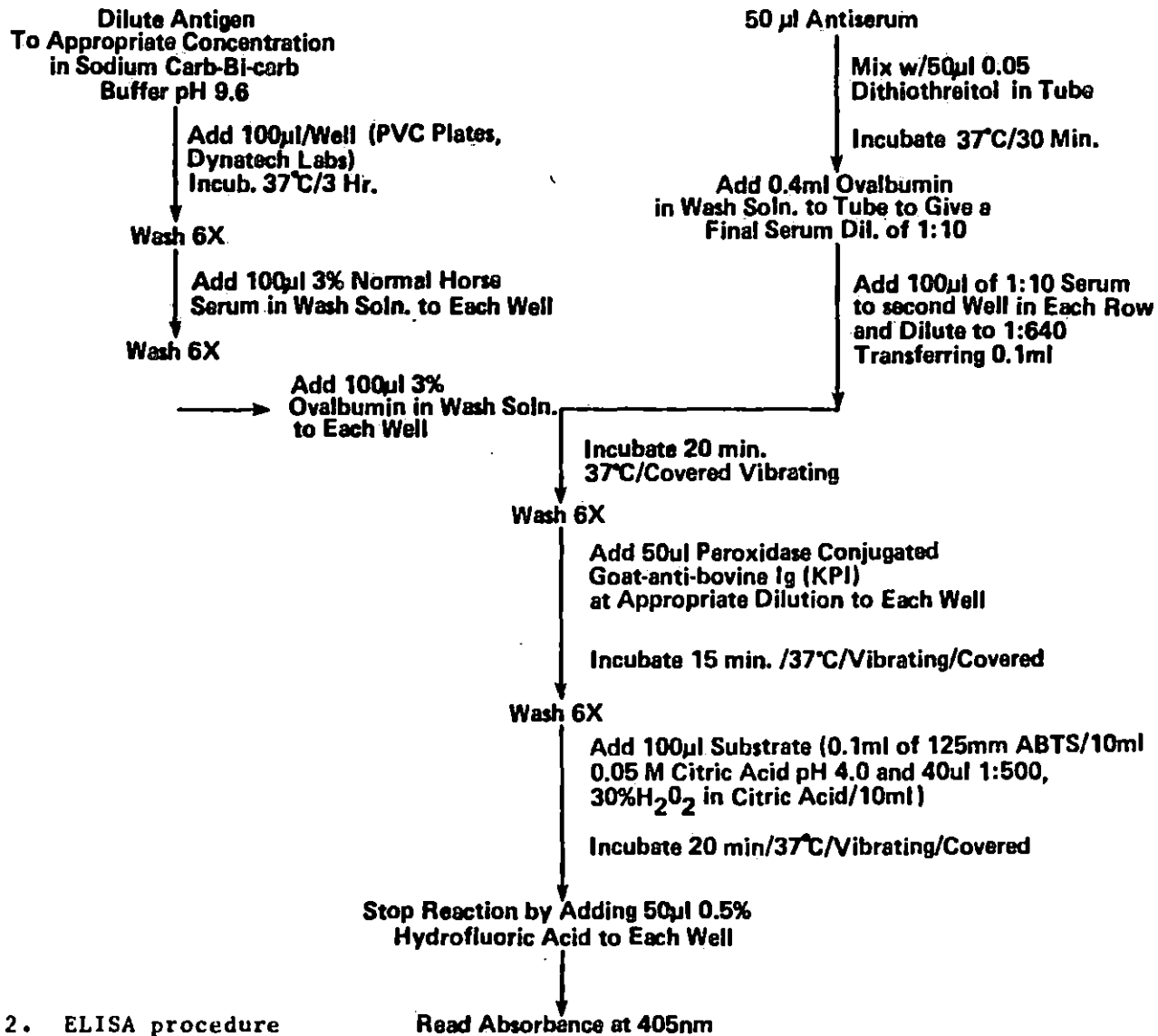
Read Absorbance at 405nm

Figure 2. ELISA procedure

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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
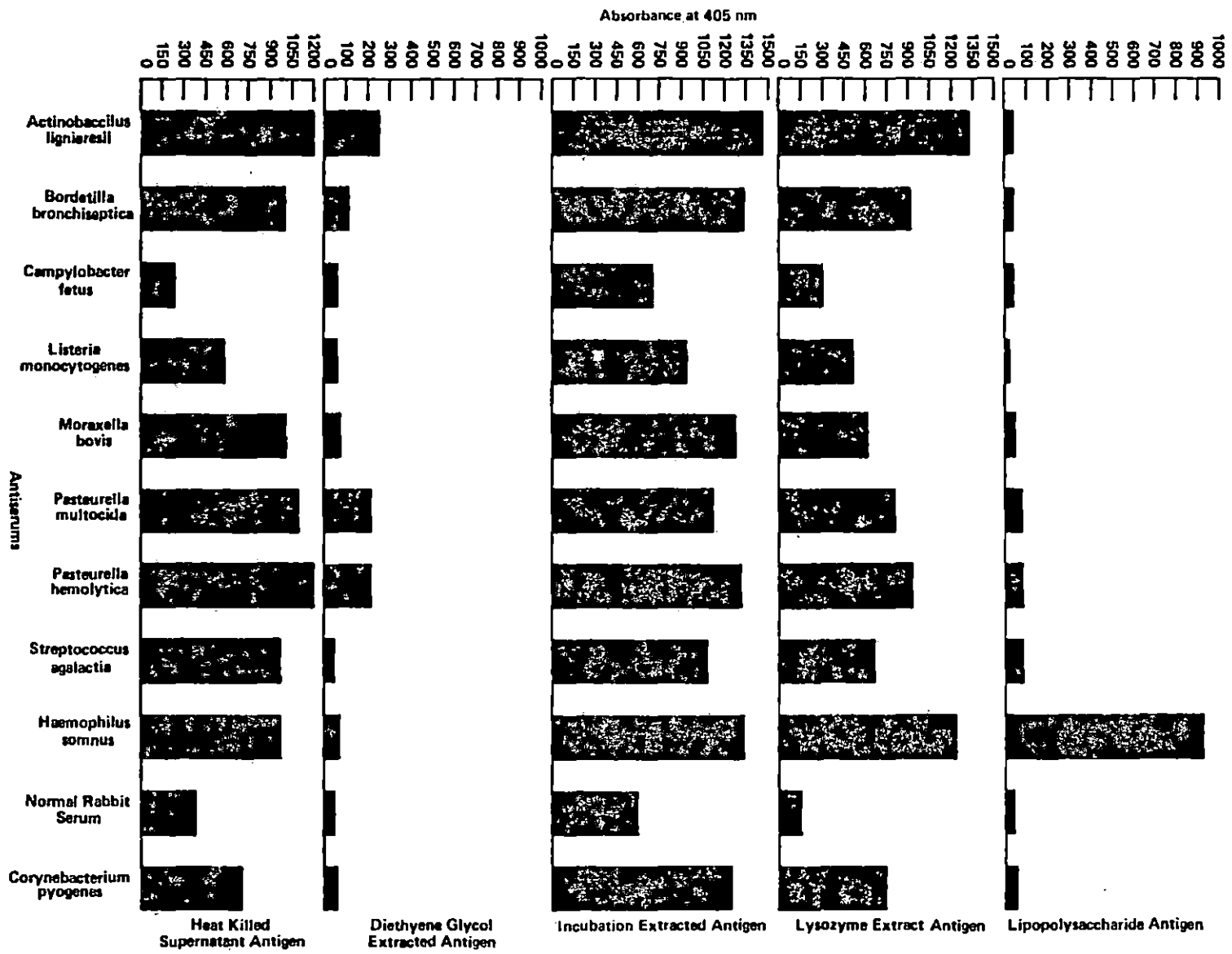
serums.


## 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

Absorbance at 405 nm



Antiserums

| | |
|---|---|
| Actinobacillus lignieresii | |
| Bordetilla bronchiseptica | |
| Campylobacter fetus | |
| Listeria monocytogenes | |
| Moraxella bovis | |
| Pasteurella multocida | |
| Pasteurella hemolytica | |
| Streptococcus agalactia | |
| Haemophilus somnus | |
| Normal Rabbit Serum | |
| Corynebacterium pyogenes | |

Heat Killed Supernatant Antigen

Diethyene Glycol Extracted Antigen

Incubation Extracted Antigen

Lysozyme Extract Antigen

Lipopolysaccharide Antigen

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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 H. somnus LPS in calf serum was determined during the peak of the 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
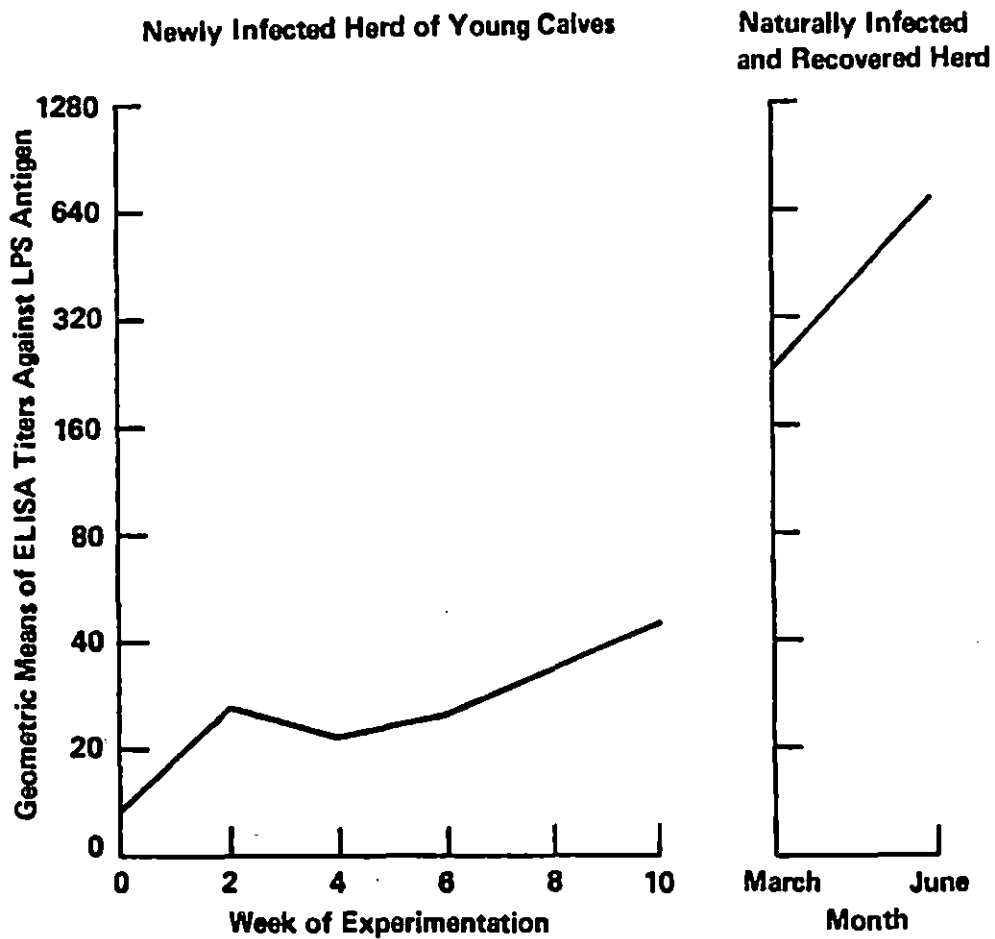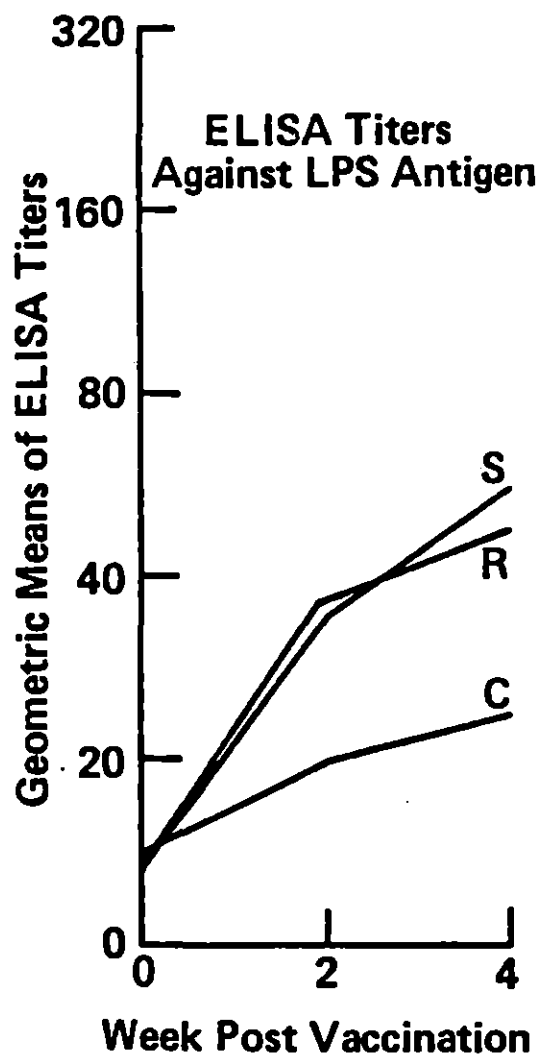
Figure 4. Comparison of the humoral antibody response
to H. somnus LPS in two separate calf herds
that had been naturally infected with H. somnus

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 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 H. somnus strain 8025 appears to be specific for 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 A. lignieresii, B. bronchiseptica, C. fetus, L. monocytogenes, M. bovis, P. multocida, P. haemolytica, S. agalactiae, and C. pyogenes, which is consistent with results of other workers which have reported common antigens associated with H. somnus and other bovine pathogens (37,48,73,91,123,136). However, contradictory reports of cross-reactions of 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
meningoencephalitis (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, H.
somnus is considered an important pathogen which causes great economic
loss to the cattle industry (118).

Lesions produced in cattle by infection with H. somnus are
characterized by the presence of large numbers of polymorphonuclear
leukocytes (PMNs). Cattle experimentally infected with 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
H. somnus may escape host defense mechanisms. Therefore the in vitro
interaction of 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% $CO_2$ 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 .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 $4.2 \times 10^8$ 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
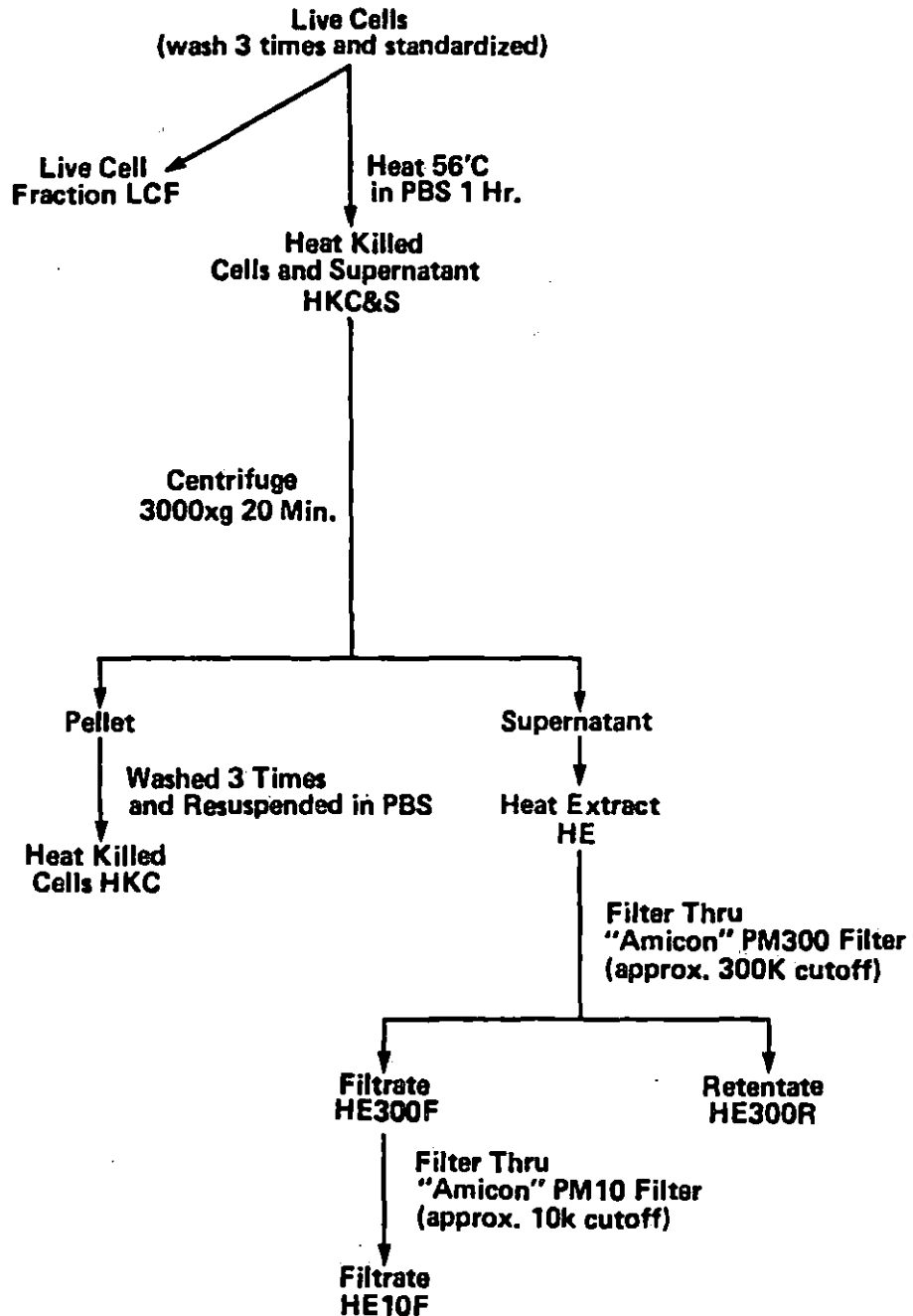
**Live Cells**
(wash 3 times and standardized)

**Live Cell
Fraction LCF**

Heat 56'C
in PBS 1 Hr.

**Heat Killed
Cells and Supernatant
HKC&S**

Centrifuge
3000xg 20 Min.

**Pellet**

Washed 3 Times
and Resuspended in PBS

**Heat Killed
Cells HKC**

**Supernatant**

**Heat Extract
HE**

Filter Thru
"Amicon" PM300 Filter
(approx. 300K cutoff)

**Filtrate
HE300F**

**Retentate
HE300R**

Filter Thru
"Amicon" PM10 Filter
(approx. 10k cutoff)

**Filtrate
HE10F**

Figure 1.   Preparation of <u>H. somnus</u> 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 $50 \times 10^6$ 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 sonication 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 $26.01 \pm 11.75$ (mean $\pm$ 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 $0.499 \pm 0.144$ (Mean $\pm$ SEF) (n = 18). None of the H. somnus fractions had a significant effect on NBT reduction (p   0.05).
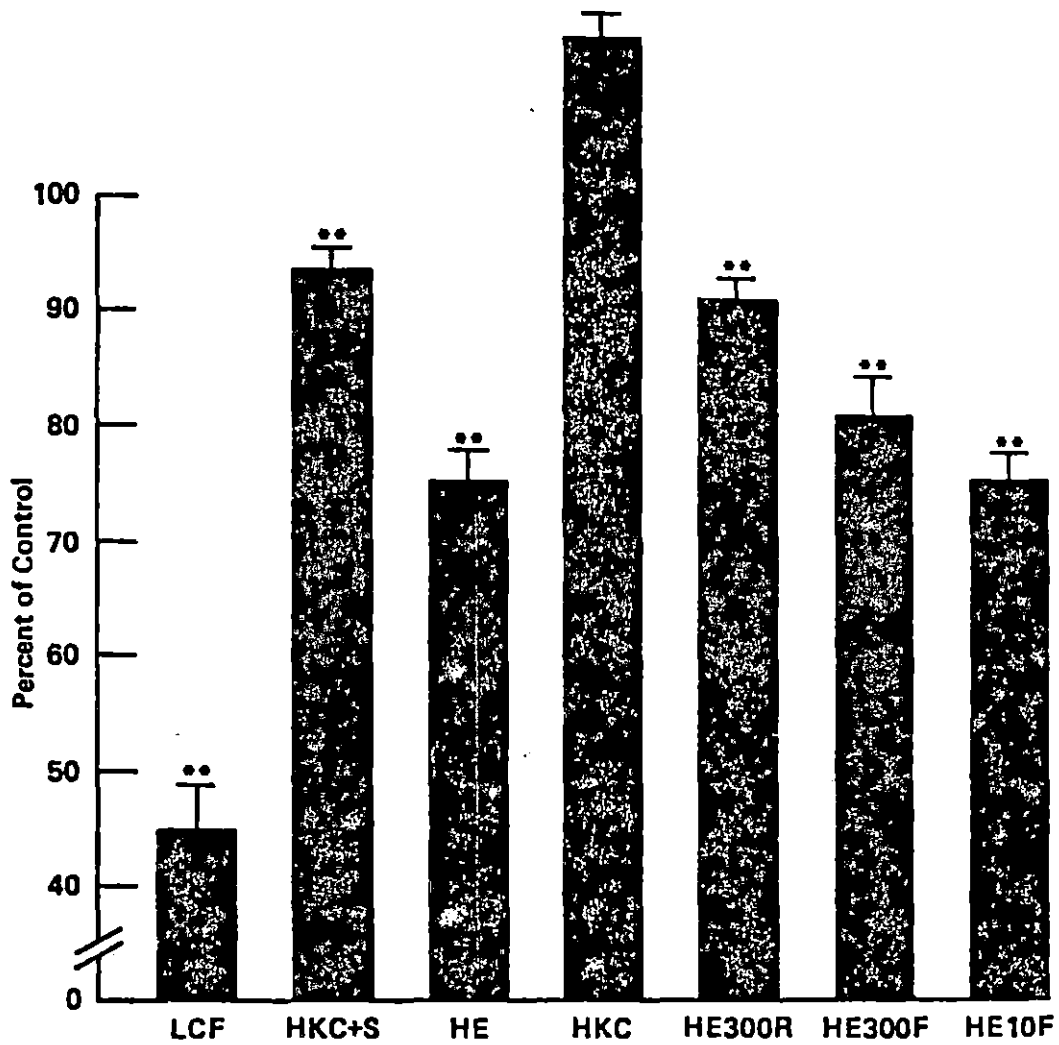
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Figure 2. Effect of H. somnus fractions on iodination by bovine
PMNs stimulated with opsonized zymosan. Values represent
mean percentages (+- standard deviation) of the control
value. Statistically significant differences from control
values are indicated as **, p < 0.05; n=16

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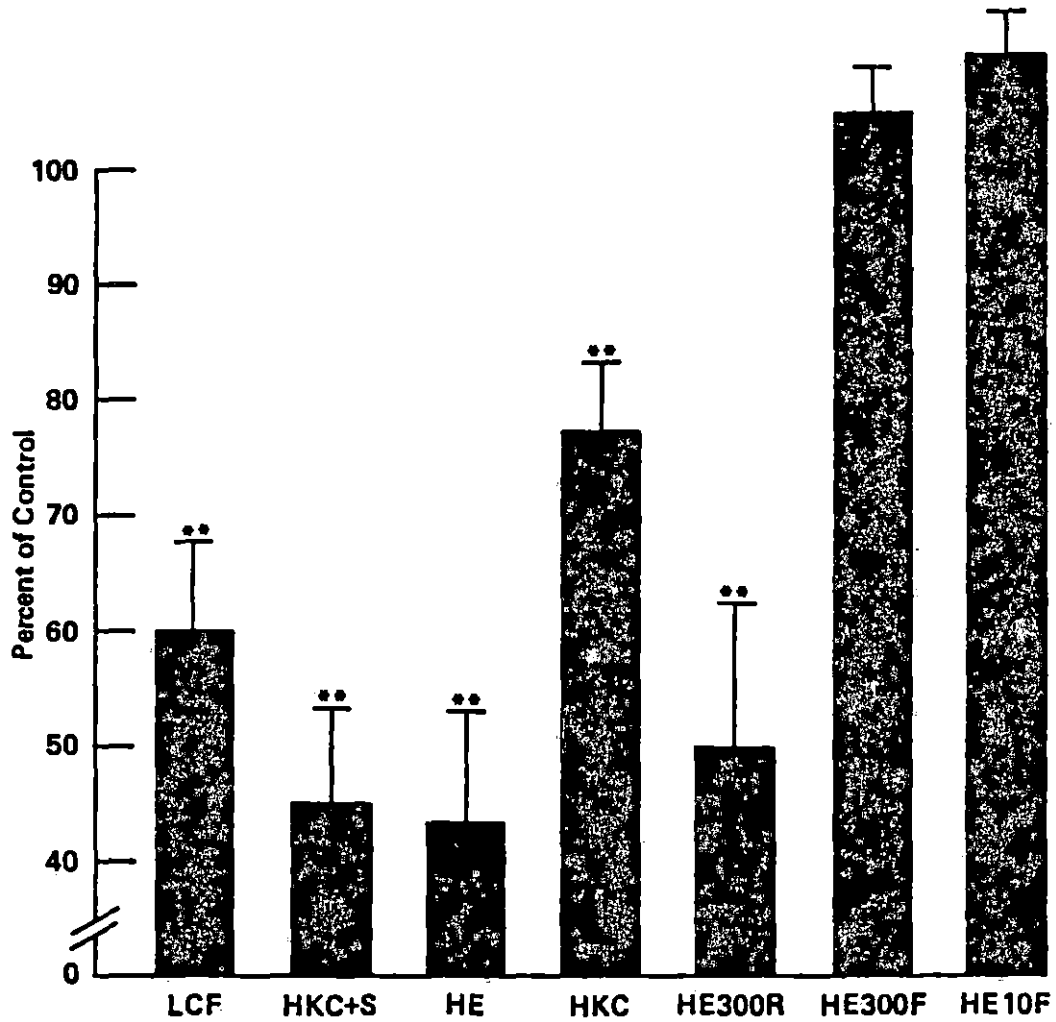


Figure 3. Effect of H. somnus fractions on S. aureus
ingestion by bovine PMNs. Values represent mean
percentages (+- standard deviation) of the control
value. Statistically significant differences from
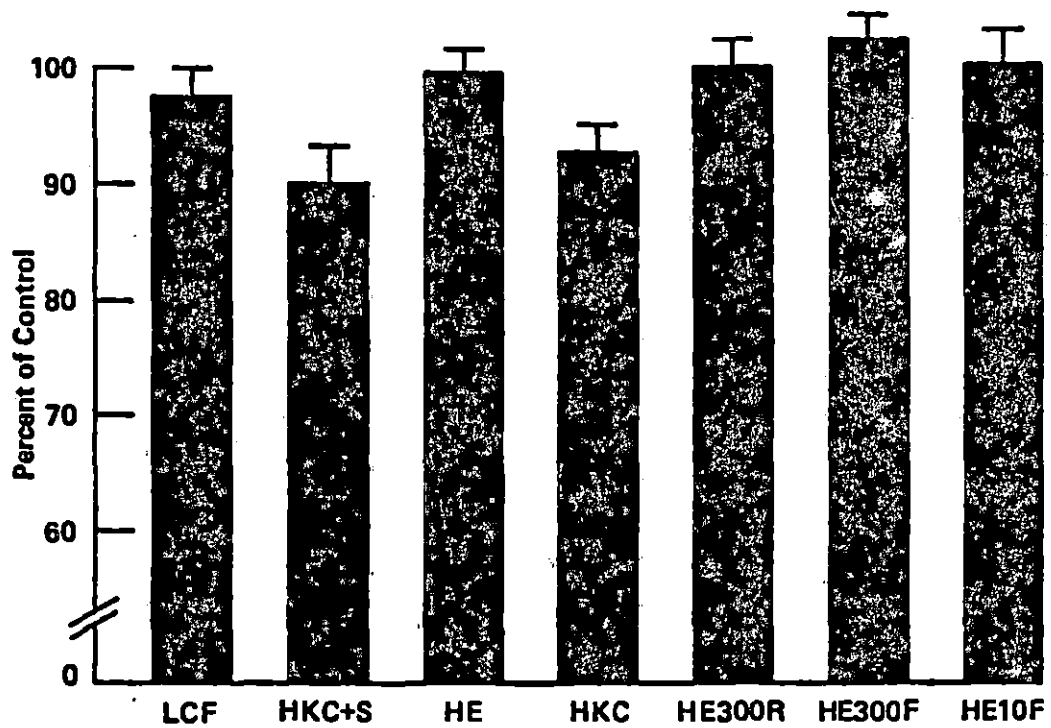control values are indicated as **, p < 0.05; n=13

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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 TEME 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.

GENERAL SUMMARY

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 antiserums 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 TEME. 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.

aureus. Inhibition of these two neutrophil functions corresonds with
the pathologic findings of H. somnus infections which occurs 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 mucocilliary
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 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 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.

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## ACKNOWLEDGMENTS

I wish to sincerely thank everyone who has helped in this experimentation and thesis preparation. First I would especially like to thank Dr. Merlin L. Kaeberle for his knowledge, patience and guidance and for providing the opportunity to accomplish this work.

I would also like to thank Dr. James A. Roth who provided assistance in performing the neutrophil assays and who computed the statistical analysis of the results. He also was a source of ideas and encouragement throughout this research.

I am grateful to Mr. Ervin Johnson for his friendship and support, Mr. Yu Wei Chiang who initiated the studies of H. somnus and the ELISA procedure and to Mr. Hyoik Ryu who assisted me in the biochemical determinations.

I am also grateful to Drs. P. A. Gough and J. Robyt who served on my graduate commitee.

I would like to express my appreciation to my wonderful wife, Robin, who continually encouraged and supported me throughout the course of my graduate study.

Finally, and most of all I would like to thank and acknowledge my Lord and Savior Jesus Christ whose grace enabled the completion of this project.

This research was supported by grant number 400-23-80 from the State of Iowa.