INTERACTIONS OF *HAEMOPHILUS SOMNUS* WITH BOVINE MACROPHAGES

A Thesis

Submitted to the College of Graduate Studies and Research

in Partial Fulfilment of the Requirements

for the Degree of

Doctor of Philosophy

in the

Department of Veterinary Pathology

Western College of Veterinary Medicine

University of Saskatchewan

Saskatoon

by

Susantha Muhandiramge Gomis

September 1995

UNIVERSITY OF SASKATCHEWAN

College of Graduate Studies and Research

Summary of Dissertation

Submitted in partial fulfilment

of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by

Susantha Muhandiramge Gomis

Department of Veterinary Pathology
University of Saskatchewan

Spring 1996

Examining Committee:

Dr. K. Post	Dean's Designate, Chair College of Graduate Studies and Research
Dr. F.A. Leighton	Chair of Advisory Committee, Department of Veterinary Pathology
Dr. G. Wobeser	Supervisor, Department of Veterinary Pathology
Dr. A.A. Potter	Co-supervisor, Veterinary Infectious Disease Organization
Dr. D. Godson	Veterinary Infectious Disease Organization
Dr. S. Sorden	Department of Veterinary Pathology
Dr. C. Riddell	Department of Veterinary Pathology

External Examiner:

Dr. C.J. Czuprynski
Department of Pathobiological Sciences
School of Veterinary Medicine
University of Wisconsin, Madison
Wisconsin 53706

INTERACTIONS OF *HAEMOPHILUS SOMNUS* WITH BOVINE MACROPHAGES

The objective of this study was to investigate the effect of *H. somnus* on phagocytosis, and bactericidal activity of bovine mononuclear phagocytes (BMP) using *in vitro* and *ex vivo* assays.

Using a flow cytometric phagocytosis assay, it was found that logarithmically growing *H. somnus* significantly inhibited the phagocytosis of opsonized *S. aureus* by bovine alveolar macrophages (BAM) obtained both from healthy calves and from cattle experimentally infected with *H. somnus*. However, neither heat- or formalin-killed, logarithmically growing *H. somnus* nor *in vitro* passaged *H. somnus* showed any effect on the phagocytic activity of these cells. In contrast to BAM, bovine blood monocytes (BBM), had a significant increase in their phagocytic activity following *in vitro* exposure to logarithmically growing *H.somnus*. The bactericidal ability of bovine mononuclear phagocytes in interaction with *H. somnus* was studied using two *in vitro* assay systems measuring nitric oxide (NO) production and chemiluminescence response. *H. somnus* rapidly inhibited the Luminol-dependent chemiluminescence (LDCL) of BBM, and of BAM costimulated with opsonized *Staphylococcus aureus*. Inhibition of the LDCL response of BBM and BAM was abrogated with either opsonized or killed *H. somnus*. In contrast to inhibition LDCL of BMP, both BBM and BAM infected with *H. somnus* had stimulated production of NO. Using a colorimetric bactericidal assay, it was found that: (1) *H. somnus* was able to survive within BBM *in vitro*

and the kinetics of its survival were similar to that seen in BBM isolated from experimentally infected cattle; (2) treatment of BBM with varying concentrations of rBoIFN-γ, rBoTNF-α, rBoIL-1β, rBoGM-CSF and *E. coli* LPS had no effect on the survival of *H. somnus*. Moreover, using ultrastructural studies, and ^{3}H uracil incorporation into nucleic acids, it was possible to demonstrate the survival of *H. somnus* in BMP. *In vitro* addition of *H. somnus* into whole blood cultures prepared from cattle experimentally infected with *H. somnus*; TNF production was below detectable level while IFN-γ and IL-6 reached a detectable level. These results indicate that the ability of *H. somnus* to modulate microbicidal activity of BMP would, in turn, assist the intracellular survival and immunopathogenesis of bovine haemophilosis.

In presenting this thesis in partial fulfilment of the requirement for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Veterinary Pathology, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4

ABSTRACT

The objective of this study was to investigate the effect of *H. somnus* on the phagocytic function and bactericidal activity of bovine mononuclear phagocytes (BMP) using *in vitro* and *ex vivo* assays.

Using a flow cytometric phagocytosis assay, it was found that logarithmically growing *H. somnus* significantly inhibited the phagocytosis of opsonized *S. aureus* by bovine alveolar macrophages (BAM) obtained both from healthy calves and from cattle experimentally infected with *H. somnus*. However, neither heat- or formalin-killed, log-phase *H. somnus* nor *in vitro* passaged *H. somnus* showed any effect on the phagocytic activity of these cells. In contrast to BAM, bovine blood monocytes (BBM) had a significant increase in their phagocytic activity following *in vitro* exposure to logarithmically growing *H.somnus*. The bactericidal ability of bovine mononuclear phagocytes in interaction with *H. somnus* was studied using two *in vitro* assay systems measuring nitric oxide (NO) production and the chemiluminescence response. *H. somnus* rapidly inhibited the Luminol-dependent chemiluminescence (LDCL) of BBM and BAM costimulated with opsonized *S. aureus*. Inhibition of the LDCL response of BBM and BAM was abrogated with either opsonized or killed *H. somnus*. In contrast to inhibited LDCL of BMP, both BBM and BAM infected with *H. somnus* had stimulated production of NO. Using a colorimetric bactericidal assay, it was found that: (1) *H. somnus* was able to survive within BBM *in vitro* and the kinetics of its survival were similar to that seen in BBM isolated from experimentally infected cattle; (2) treatment of BBM with varying concentrations of rBoIFN-γ, rBoTNF-α, rBoIL-1β,

rBoGM-CSF and *E. coli* LPS had no effect on the survival of *H. somnus*. Moreover, using ultrastructural studies, and ^{3}H uracil incorporation into nucleic acids, it was possible to demonstrate the survival of *H. somnus* in BMP. With *in vitro* addition of *H. somnus* to whole blood cultures prepared from cattle experimentally infected with *H. somnus*, TNF production was not detectable during the 24 h incubation period while IFN-γ and IL-6 were detected. These results indicate that the ability of *H. somnus* to modulate microbicidal activity of BMP could, in turn, assist the intracellular survival and contribute to pathogenesis of bovine haemophilosis.

ACKNOWLEDGEMENTS

My sincere thanks are extended to my supervisor Dr. Gary Wobeser and co-supervisors Drs. Andrew Potter and Dale Godson for their advice, encouragement, patience and guidance throughout this project. Thanks are also extended to my committee members Drs. F.A. Leighton, J. Orr, H. Hughes, and S. Sorden for their encouragement and advice throughout my studies. A thank you is also extended to Dr. Lorne Babiuk for providing me an opportunity to carry out my studies at V.I.D.O.

I would like to thank individuals at V.I.D.O. for their support and friendship. Special thanks to Dr. W. Chen for his assistance with some of the experimental designs; to Drs. Richard Harland, Joyce Van Donkersgoed, and Trent Watts for their support in the bovine experimental studies; Terry Beskorwayne and Noreen Rapin for their expertise with the flow cytometer; Barry Carroll, Brock Evans, Jason Peters, Cindy Toy, Heidi Seida, Gail McLeod and Leona Boyer for their support in daily laboratory work. I would like to thank individuals at the Department of Veterinary Pathology especially Ian Shirley and Kathie Caspell for the support in electron microscopic studies; Ed Bueckert and his staff for their support in preparation of histological slides; Lois Ridgway and Colleen Dembiczak for their assistance in necropsy procedure. A thank you is also extended to Dr. D. Haines and her staff at Department of Veterinary Microbiology for immunohistochemical studies. A special thank to Dr. Manjula Bandara (Department of Horticulture Science, University of SK.) for his advice and assistance in statistical analysis.

Financial support for my research was provided by V.I.D.O. Personal support was provided by a scholarship from Canadian Commonwealth and Fellowship Plan.

I am indebted to those organizations for providing me with the opportunity to pursue my studies.

I would like to thank for my mother for her support and encouragement. Finally, I would like to extend my sincere thanks to my wife, daughter and son for their understanding and constant support through my studies.

DEDICATION

To my late father, Muhandiramge Buddadasa Gomis and to my late brother, Muhandiramge Moulie Gomis.

TABLE OF CONTENTS

LIST OF FIGURES

1. LITERATURE REVIEW

1.1 Classification and Morphology of *Haemophilus somnus*

Haemophilus somnus is a Gram-negative, catalase-negative, poorly fermentative, highly pleomorphic, coccobacillus. It ranges in size from 0.3-0.5 μm by 0.8-4.0 μm and can be found as single cells, doublets, or in long filamentous chains (Kennedy *et al.*, 1960; Glossing, 1966; Bailie, 1969; Humphrey *et al.*, 1982a; Humphrey and Stephens, 1983). The organism is non-piliated, non-sporeforming, non-capsulated, and non-motile but does produce a weak haemolysin (Gossling, 1966; Stephens and Little, 1981; Humphrey and Stephens, 1983). Colonies of *H. somnus* grown under optimal conditions are convex, circular, entire, moist and glistening, and slight yellow or grey-yellow colour, reaching a size of 1-2 mm in 2-3 days (Gossling, 1966; Garcia-Delgado *et al.*, 1977). The intensity of the colour ranges from a light yellow to almost orange with the deepest colour being seen from fresh isolates (Corboz, 1981; Humphrey, 1982). The yellow colour of the colonies is especially evident when colonies are raised on a bacteriological loop (Williams *et al.*, 1978). Older colonies may become granular with a pilliate centre and flattening of the periphery (Kennedy *et al.*, 1960; van Dreumel *et al.*, 1970). *H. somnus* is a fastidious organism that requires rich media for growth in the laboratory (Inzana and Corbeil, 1987). Optimal growth was found on incubation in an atmosphere of 10% CO_2, 37C (Gracia-Delgado *et al.*, 1977). *H. somnus* has an absolute growth requirement for uracil, but not for another bases (Inzana and Corbeil, 1987).

Ultrastructural studies showed that the cell envelope of *H. somnus* is similar to the envelope of other Gram-negative bacteria (Stephens and Little, 1981) and is therefore likely to contain endotoxic lipopolysaccharide (LPS). Electrophoretic analysis however, demonstrated that *H. somnus* endotoxin was a lipooligosaccharide (LOS), due to the lack of high-molecular-weight repeating O-side-chain oligosaccharides (Inzana *et al.*, 1988). Although, the placement of this organism is within the genus *Haemophilus*, *H. somnus* does not require X (haemin) and V (nicotinamide adenine dinucleotide) factors for its growth (Shigidi, 1970; Biberstein, 1981; Stephens *et al.*, 1983). The demonstration that *H. somnus* is able to synthesize porphyrins from amino levulinic acid firmly established its X factor independence (Biberstein, 1981). This *Haemophilus*-like organism remains in the genus since no firm taxonomic position has been reached as to which group it would fit, as well as (a) its DNA has a G/C content within 1% of *Haemophilus influenzae* (Bailie *et al.*, 1973; Walker *et al.*, 1985); and (b) it has many similarities with other organisms within the family *Pasteurellaceae* (*Haemophilus*, *Actinobacillus*, and *Pasteurella*) (Walker, 1985) which include penicillin-binding proteins (Mendelman and Serfass, 1988), cross reactive antigens (Kennedy *et al.*, 1960; Miller *et al.*, 1975; Canto *et al.*, 1983; Corbeil *et al.*, 1987), and similar biochemical properties (Kennedy *et al.*, 1960; Stephens *et al.* 1983).

1.1.1 Disease manifestations of Haemophilosis

Historically, the major *H. somnus* problem was considered to be the nervous system disease variously called thrombotic meningoencephalitis (TME); thromboembolic meningoencephalitis (TEME); infectious thromboembolic

meningoencephalitis (ITEME); or sleeper syndrome (Griner *et al.*, 1956; Kennedy *et al.*, 1960; Bailie *et al.*, 1966; Bailie *et al.*, 1973; MacDonald *et al.*, 1973; Humphrey, 1982; Blood *et al.*, 1983; Little, 1986; Harris and Janzen, 1989). However, fewer cases of TME are being encountered or reported each year. Instead, other disease conditions, such as pneumonia, and myocarditis are becoming more prevalent (Schuh and Harland, 1991; Orr, J.P. 1992). Because there are many different syndromes, they have been referred to as the "*Haemophilus somnus* disease complex" or "haemophilosis" (Janzen *et al.*, 1981; Humphrey and Stephens, 1983; Corbeil *et al.*, 1985; Little, 1986; Harris and Janzen, 1989).

Although the reproductive tract is considered to be the ecological niche or reservoir of *H. somnus* (Humphrey *et al.*, 1982b; Little, 1986), except for infrequent reports of infertility (Von Luginbuhl and Kupfer, 1980) and poor semen quality, the organism does not seem to cause disease in bulls and steers in these sites (Janzen *et al.*, 1981; Humphrey *et al.*, 1982a; Humphrey *et al.*, 1982b). In female cattle, *H. somnus* can cause vaginitis, endometritis, infertility, and abortion (Patterson *et al.*, 1984; Stephens *et al.*, 1986). Calves from infected cows have been born weak or stunted and can die shortly after birth (Waldham *et al.*, 1974; Cain, 1985; Cain and Dennis, 1987). *H. somnus* has also been isolated from the nasal cavities (Brown *et al.*, 1970; Crandell *et al.*, 1977; Hall *et al.*, 1977) and trachea (Corstvert *et al.*, 1973) of normal cattle.

H. somnus can gain entry to the circulatory system and cause septicaemia (Humphrey, 1982; Blood *et al.*, 1983; Little, 1986; Harland *et al.*, 1990). *H. somnus* is known to localize in the brain, heart, skeletal muscle, joints, larynx, liver, and

kidneys (Kennedy *et al.*, 1960; Weide *et al.*, 1964; Bailie *et al.*, 1966; Panciera *et al.*, 1968). Localization can, and usually does, occur simultaneously in one or more sites (Harris and Janzen, 1989).

The respiratory form of *H. somnus* infection has gained in importance. In the upper respiratory tract, *H. somnus* can cause laryngitis as well as tracheitis (Dillman, 1972). Many clinical descriptions of the disease report that lower respiratory tract disease often precedes outbreaks of TEM (Weide *et al.*, 1964; Panciera *et al.*, 1968; Dillman, 1972; ; Saunders *et al.*, 1980; Ames, 1987). In case control studies, *H. somnus* has been isolated in pure culture from as many as 28% of the cases of pneumonic lungs examined microbiologically (Groom, 1985). *H. somnus* alone is capable of causing bronchopneumonia (Andrews *et al.*, 1983; 1984; Groom, 1985; Andrews *et al.*, 1985). *H. somnus* can cause severe fibrinous pleuritis (Weide *et al.*, 1964; Panciera *et al.*, 1968; Saunders *et al.*, 1980; Groom, 1985), which may also rarely be found in conjunction with fibrinous pneumonia.

Cases of chronic arthritis characterized by excessive joint fluid and fibrinous clots have been reported (Panciera *et al.*, 1968; Humphrey, 1982; Harris and Janzen, 1989; Harland *et al.*, 1990). In addition, there may be congestion, edema, and petechial haemorrhages of the synovial membranes (Panciera *et al.*, 1968).

Otitis has been reported in some feedlot cattle in which a copious clear yellow fluid draining from the ears of febrile animals yielded pure cultures of *H. somnus* (Nation *et al.*, 1983).

Pure cultures of *H. somnus* have been obtained from cases of conjunctivitis in herds with no previous history of either respiratory disease or TME (Lamont and Hunt,

1982; Nation *et al.*, 1983).

Chronic mastitis has been reported to occur spontaneously in both North America and Europe (Armstrong *et al.*, 1986; Higgins *et al.*, 1987). Chronic and gangrenous mastitis have been produced experimentally in dairy cows (Hazlett *et al.*, 1983). Cultures of the organism has usually been restricted to one quatre. Milk production was almost nonexistant and the secretion varied from blood-tinged and watery with small fibrin clots (Armstrong *et al.*, 1986) to white, homogenous and without evidence of blood (Higgins *et al.*, 1987).

H. somnus has been known to survive in various bovine secretions. Isolates have been cultured from urine (Dewey and Little, 1984), semen and seminal fluids (Humphrey *et al.*, 1982b), vaginal discharges (Dewey and Little, 1984; Kwiecien and Little, 1992), feces (Dewey and Little, 1984), nasal mucous (Crandell *et al.*, 1977), blood (Dewey and Little, 1984), and cerebral spinal fluid (Dewey and Little, 1984). Although growth and prolonged survival of the bacterium have been shown in blood, nasal mucous and vaginal mucous, prolonged survival in other fluids has only been shown to occur at -70C (Dewey and Little, 1984; Harris and Janzen, 1989).

1.1.2 Pathogenesis

The reproductive tract is considered the most likely reservoir site of *H. somnus* (Humphrey *et al.*, 1982a,b). Females and males harbour *H. somnus* within their reproductive or urinary tracts and can shed the organism in urine or discharges to contaminate the environment (Janzen *et al.*, 1981; Humphrey *et al.*, 1982a,b). In bulls, as well as steers, *H. somnus* is commonly isolated from the prepuce. It can also be

recovered from the bladder, accessory sex glands and ampullae (Humphrey *et al.*, 1982a; Humphrey *et al.*, 1982b; Corbeil *et al.*, 1986). *H. somnus* does not survive in the environment for more than 2 h in urine, but this route of transmission as an aerosol or aspirate may be important (Dewey and Little, 1984). It is also thought that routes of infection other than the respiratory tract, such as Batson's veins which course directly along the spinal column to the brain from the reproductive tract, are possible (Dommisse, 1975; Miller *et al.*, 1983).

Once *H. somnus* has localized in tissues, it causes endothelial cells of small blood vessels to separate and thus expose the underlying basement membrane. This activates the coagulation mechanism and results in the formation of a thrombus (Thompson and Little, 1981); hence the name "thrombo" that is sometimes given to the disease. The name TEME was likely truncated to thrombotic meningoencephalitis (TME) after noticing that there was no evidence of embolic events in the pathogenesis of the disease (Stephens *et al.*, 1981). It is now considered that the lesion is an *in situ* thrombosis and not a thromboembolism. Interruption of the blood supply results in destruction of tissues and development of clinical signs (Harris and Janzen, 1989).

In vitro studies have established that bovine neutrophils are unable to kill *H. somnus*, and that *H. somnus* can replicate within bovine monocytes (Lederer *et al.*, 1986). Additional studies demonstrated that bovine alveolar macrophages and blood monocytes ingest but do not kill opsonized *H. somnus*. These findings suggest that the bacterium can persist and proliferate within these cells and contribute to the pathogenesis of haemophilosis (Lederer *et al.*, 1986).

1.1.3 Virulence factors of *Haemophilus somnus*

Although there is no major cultural, biochemical, electrophoretic or serological variation among isolates (Gossling, 1966; Gracia-Delgato *et al.*, 1977; Humphrey *et al.*, 1982a) there appear to be differences in virulence (Humphrey *et al.*, 1982b; Groom *et al.*, 1988) and biotypes may exist within the species *H. somnus* (Humphrey *et al.*, 1982a; Humphrey *et al.*, 1982b). Strain differences in virulence have been demonstrated after intravenous, intracisternal or intratracheal inoculation of *H. somnus* (Humphrey and Stephens, 1983; Jackson *et al.*, 1987; Groom *et al.*, 1988). Groom *et al.*, (1988) suggested that isolates from cases of pneumonia were more virulent than encephalitic strains, which in turn were more virulent than bacteria isolated from the prepuce of bulls identified as carriers. *H. somnus* isolated from the prepuce or semen were generally not pathogenic, even following intratracheal challenge of calves (Humphrey, 1982; Groom *et al.*, 1988). Kwiecien and Little (1992) found that genital isolates from slaughtered cows had pathogenic potential. They determined that more than half of the *H. somnus* isolates produced lesions after intracerebral-spinal challenge. Since pathogenic and non-pathogenic strains of *H. somnus* can be isolated from reproductive and respiratory tract of clinically normal animals, it is not clear what host or microbial factors are responsible for this host-parasite relationship.

Recently, methods have been developed that differentiate between pathogenic and nonpathogenic bacteria. Yarnall and Corbeil (1989) have identified nonspecific immunoglobulin binding proteins (Fc receptors) of 41 KDa and 270 KDa that appear to only be associated with virulent bacteria. These proteins have been used in serological tests to identify animals that were either carriers or noninfected and those

that had been exposed to pathogenic *H. somnus* and were now immune (Widders *et al.*, 1986; Yarnall *et al.*, 1988a,b; Widders *et al.*, 1989a,b; Yarnall and Corbeil, 1989). Susceptibility to killing by complement has also been used to differentiate between isolates of different virulence: serum resistant isolates were considered to be virulent while serum sensitive isolates were not (Corbeil *et al.*, 1985).

Bacterial Fc receptors have been found to be secreted in conjunction with outer membrane blebs. These Fc receptors show a specificity for bovine IgG_2, IgA, and IgM (Yarnall *et al.*, 1988a,b; Yarnall and Corbeil, 1989). Transferrin receptors have also been identified on the surface of *H. somnus* (Yu *et al.*, 1992; Ogunnawiro *et al.*, 1990).

The LOS possesses typical endotoxic activity and may contribute to the vascular endothelial damage and other lesions associated with *H. somnus* infection (Corbeil *et al.*, 1986; Gogolewski *et al.*, 1987b; Inzana *et al.*, 1988; Nakajima and Ueda, 1989; Corbeil, 1990; Fenwick, 1990; Silva and Little, 1990). Intrastrain instability or phenotypic phase variation of LOS of *H. somnus* occurs during infection and *in vitro* passage. (Inzana *et al.*, 1992).

H. somnus has been shown to possess the ability to attach to different bovine cells *in vitro* including epithelial surfaces of the turbinates (Ward *et al.*, 1984), bovine embryos (Thomson *et al.*, 1988), sperm (Chelmonska, 1990) and cultured endothelial cells (Thompson and Little, 1981). Necrosis and sloughing of cells were observed with various endothelial cultures, indicating that *H. somnus* may be producing a toxic product (Thompson and Little, 1981; Gogolewski *et al.*, 1987a).

It has been reported that *H. somnus* is able to survive in bovine neutrophils and also that this interaction is associated with the inhibition of many neutrophilic activities

(Pennell and Renshaw, 1977; Czuprynski and Hamilton, 1985; Chiang *et al.*, 1986). Phagocytosis and the myeloperoxidase-hydrogen peroxide-halide system have been reported to be inhibited by the *in vitro* release of purine nucleotides and nucleosides following exposure of the phagocytes to *H. somnus*. However, it appears that other mechanisms also play an important role in altering phagocyte function (Chiang, *et al.*, 1986; Hubbard, *et al.*, 1986; Sample and Czuprynski, 1991). Actual growth of *H. somnus* has been observed within alveolar macrophages and blood monocytes (Lederer *et al.*, 1987), resulting in visible damage and eventual death of the phagocytes. Some workers (Thompson and Little, 1981; Gogolewski *et al.*, 1987a; Gogolewski *et al.*, 1989) have shown that *H. somnus* is toxic to alveolar macrophages *in vitro* and *in vivo*. Specific toxins have not been identified. The precise mechanisms allowing for intracellular survival and growth remain to be elucidated. *H. somnus* is known to be a catalase-negative organism yet it was recently reported by Sample and Czuprynski (1991) that viable *H. somnus* were able to remove hydrogen peroxide from solution in an energy-dependent manner.

1.2 Bacterial:Phagocyte Interactions

1.2.1 Phagocytosis

Phagocytosis represents an important effector mechanism for the eradication of infectious agents. It is performed primarily by specialized cells of two different lineages, namely polymorphonuclear neutrophilic granulocytes (PMN), and mononuclear phagocytes (MP). Because of their function, PMN and MP are often referred to as "professional phagocytes" to distinguish them from most other host cells

which are called "non-professional phagocytes" (Kaufmann and Reddehase 1989).

Two forms of phagocytosis have been identified: conventional phagocytosis and coiling phagocytosis. In conventional phagocytosis, phagocyte pseudopodia move circumferentially and more or less symmetrically around the organism and fuse at the distal side. In coiling phagocytosis, phagocyte pseudopodia coil around the organism as it is internalized (Horwitz, 1984). *Toxoplasma gondii* (Jones *et al.*, 1972), *Trypanosoma cruzi* (Tanowitz *et al.*, 1975; Nogueira and Cohn, 1976), and *Mycobacterium tuberculosis* (Payne *et al.* 1987) enter phagocytes by conventional phagocytosis; *Legionella pneumophila* (Horwitz, 1984) and, apparently, *Leishmania donovani* (Chang, 1979) enter by coiling phagocytosis.

Professional phagocytes are among the major effector cells of the anti-infection defence. Phagocytosis alone cannot eradicate invading microbes; it must be followed by intracellular killing mechanisms. To fulfil these functions, PMNs and MP have specific receptors for microorganisms and specialized organelles and molecules for killing and degrading them. PMNs interact with antibodies and complement alone, while MP are also controlled by T lymphocytes. A major difference between PMNs and MP is their lifespan; PMN live for few days and MP often last for many weeks. Thus, cells of the monocyte lineage represent particularly mobile elements capable of traversing different compartments of the body, providing a means for the dissemination of pathogens from the site of invasion to the most appropriate tissue site. It is perhaps for these reasons that many pathogens have chosen professional phagocytes as their major targets (Kaufmann, and Reddehase, 1989).

1.3 Receptor Mediated Phagocytosis

1.3.1 Complement Receptor Mediated Phagocytosis

Complement receptors on mouse macrophages have been shown to mediate ingestion of *Leishmania donovani* (Blackwell *et al.*, 1985) and *Leishmania major* (Mosser and Edelson, 1985), and complement receptors on human monocytes have been shown to mediate ingestion of *Legionella pneumophila* (Payne and Horwitz, 1987), *Mycobacterium tuberculosis* (Payne *et al.*, 1987), *Mycobacterium leprae* (Schlesinger and Horwitz, 1988), and *Histoplasma capsulatum* (Bullock and Wright, 1987).

Both complement receptor CR1, which recognizes complement component C3b (Wilson *et al.*, 1987) and CR3, which recognizes complement component C3bi (Wilson *et al.*, 1987), have been shown to mediate phagocytosis of intracellular parasites. In the presence of fresh serum, CR3 has been found to mediate the uptake of *Leishmania donovani* (Blackwell *et al.*, 1985; Wilson, and Pearson, 1988), *Leishmania major* (Mosser and Edelson, 1985), *Legionella pneumophila* (Payne and Horwitz, 1987), *Mycobacterium tuberculosis* (Payne *et al.*, 1987), *Mycobacterium leprae* (Schlesinger and Horwitz, 1988; Schlensinger, and Horwitz, 1990), *Yersinia pseudotuberculosis* (Isberg, and Leong, 1990) and *Histoplasma capsulatum* (Bullock and Wright, 1987).

In the presence of serum, the major ligand on the parasite recognized by CR3 is presumably C3bi; in the absence of serum, the identity of the ligand recognized by CR3 is unclear. It is also possible that the ligand is still C3bi, derived not from serum but directly from the mononuclear phagocyte. Human monocytes and alveolar macrophages generate the components of functional alternative pathway (Hetland and

Eskeland, 1986; Hetland *et al.*, 1986) and functional classical pathway (Hetland *et al.*, 1987) of complement activation, and particles incubated with monocyte supernatants fix C3 (Ezekowitz *et al.*, 1984). It is also possible that the ligand for CR3 is a parasite component recognized by a lectin-like or adhesion-promoting domain of the CR3 receptor (Ross *et al.*, 1985).

The complement receptor pathway may provide intracellular parasites with safe passage into mononuclear phagocytes. Binding to CR1 or CR3 causes endocytosis but does not induce reactive oxygen intermediates ((Detmers and Wright, 1988). Ligation of CR1 and CR3 receptors by particles coated with C3b or C3bi does not consistently stimulate the release of oxygen metabolites, such as hydrogen peroxide, or superoxide or the release of mediators of inflammation such as metabolites of arachidonic acid (Wright and Silverstein, 1983; Yamamoto and Johnston, 1984; Aderem *et al.*, 1985). Thus, complement coated particles entering by the complement receptor pathway may avoid the adverse consequences of the respiratory burst. Consistent with this hypothesis, *Legionella pneumophila* (Jacobs *et al.*, 1984), *Mycobacterium tuberculosis* (Horowitz, 1988), *Mycobacterium leprae* (Holzer *et al.*, 1986), and *Toxoplasma gondii* (Wilson *et al.*, 1980) have been found to elicit little or no metabolic burst upon entering mononuclear phagocytes. Particles that are ingested via CR3 do not trigger superoxide anion production (Yamamoto, and Johnston, 1984). In this regard, the nature of the parasite surface ligands recognized by the CR3 receptor may be critical. In contrast to the situation with C3b and C3bi-coated particles, stimulation of CR3 with β-glucan particles triggers a strong oxidative burst (Ross *et al.*, 1987; Cain *et al.*, 1988). Thus, a parasite interacting with the postulated lectin-like domain of the CR3

receptor, rather than the C3bi binding domain, may trigger an oxidative burst. Because intracellular predators often enter their prey without inducing reactive oxygen metabolites, their growth in activated macrophages must ultimately be controlled by non-oxidative mechanisms (Flesch and Kaufmann, 1988; Kagaya, 1989; Wolf, 1989; Mor, 1989).

Recent *in vivo* studies indicate that CR3 on mononuclear phagocytes is involved in the accumulation of MP at sites of *Listeria monocytogenes* replication (Rosen *et al.*, 1989). Thus, CR3 may be used not only in the entry of many intracellular pathogens into professional phagocytes, but may also play a role in accumulation of MP at sites of bacterial growth.

1.3.2 Mannose Receptor Mediated Phagocytosis

Tissue macrophages express mannose receptors, while circulating phagocytes lack them. However, there is a soluble mannose-binding protein in the circulation which can act as opsonin for mannose rich bacteria (Kuhlman, *et al.*, 1989). Monocyte derived macrophages express mannose receptors that recognize mannose and fucose (Stahl, 1990). Mannose receptors have been found to play a role in mediating phagocytosis of intracellular pathogens (Blackwell *et al.*, 1985; Wilson and Pearson, 1988; Bermudez *et al.*, 1991; Roecklein *et al.*, 1992). The mannosyl-fucosyl receptor mediates adherence of *Leishmania donovani*, at least under serum free conditions (Blackwell *et al.*, 1985; Wilson and Pearson, 1988).

1.3.3 Fibronectin Receptor Mediated Phagocytosis

Fibronectin is a glycoprotein that binds to cell surfaces. Many bacteria can bind fibronectin, a property which facilitates bacterial colonization of host tissue. Professional phagocytes express fibronectin receptors (FnR) and thus fibronectin can also act as an opsonin. Receptors for fibronectin may play a role directly, or indirectly by virtue of the capacity of fibronectin to upregulate mononuclear phagocyte complement and Fc receptors (Bevilacqua *et al.*, 1981; Pomier *et al.*, 1983; Wright *et al.*, 1984; Brown and Googwin, 1988). Some intracellular pathogens possess fibronectin binding activity, which suggests that they use FnR in order to enter phagocytes. For example, *Mycobacterium tuberculosis* secrete large quantities of fibronectin-binding molecules of 30-32KD and 57-60KD (Abou-Zeid *et al.*, 1988). Fibronectin has also been reported to bind to *Leishmania mexicana* promastigotes and amastigotes, *Leishmania tropica* amastigotes, and *Trypanosoma cruzi* trypamastigotes, and to enhance binding of *Leishmania* to human monocytes and *Trypanosoma cruzi* to mouse macrophages and rat fibroblasts (Wirth and Kierszenbaum, 1984; Ouaissi *et al.*, 1984; Wyler *et al.*, 1985).

1.3.4 Fc Receptor Mediated Phagocytosis

Binding of IgG-coated particles induces endocytosis and triggers multiple biological activities, including the production of reactive oxygen metabolites (Mellman, 1988). CR1 and CR3 provide a much safer way than does FcR for entry into professional phagocytes.

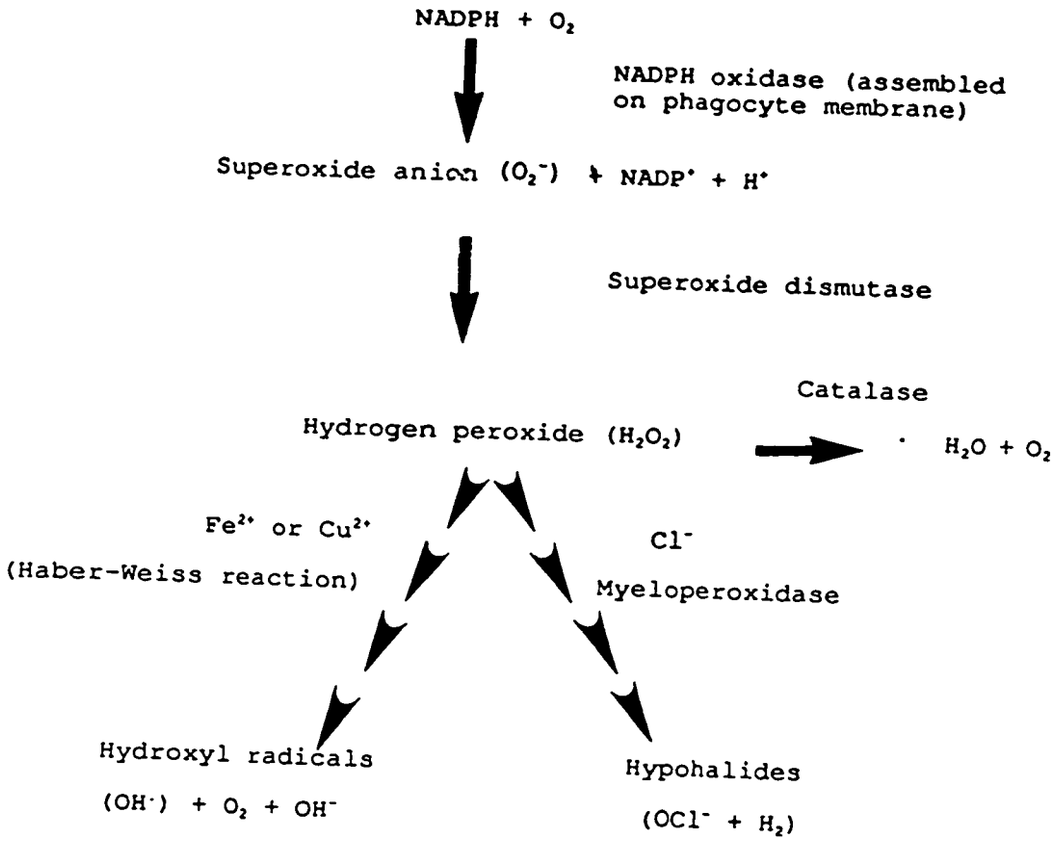
1.4 Microbial Killing Mechanisms of Phagocytes

1.4.1 *In Vitro* Generation of Reactive Oxygen Species by Phagocytes.

The production of reactive oxygen species via activation of the nicotinamide-adenine dinucleotide phosphate NADPH oxidase of phagocytes is known as the respiratory burst (Babior, 1978; Klebanoff, 1980; Berton *et al.*, 1988) (Fig. 1). Biochemically, the burst is characterized by rapid uptake of oxygen, enhanced metabolism of glucose, and release of a wide variety of reactive oxygen intermediates (Badwey and Karnovsky, 1980; Babior, 1978). The consumption of oxygen follows the activation of a membrane-bound reduced NADPH oxidase (Nathan and Tsunawaki, 1984). Glucose is metabolized principally through the hexose monophospate shunt (HMP), with minor amounts oxidized via the oxidative phosphorylative pathways (Romeo *et al.*, 1973; Montarroso and Myrvik, 1979; Papermaster *et al.*, 1980;). A large portion of the oxygen is used for energy metabolism, but substantial amounts also are reduced to superoxide anion (Roos and Van der Stijl-Neijenhuis, 1980; Rossi *et al.*, 1980). Once generated, the anion spontaneously or catalytically dismutates to hydrogen peroxide. Superoxide and hydrogen peroxide then interact to generate an array of reactive derivatives of molecular oxygen, which includes superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) (Badwey and Karnovsky 1980; Klebanoff, 1980). The expression of oxidase activity is the end result of a rapid activation of a variety of incompletely defined pathways (McPhail and Snyderman, 1984; Tauber, 1987).

The NADPH oxidase is positioned in the membrane so that reactive oxygen

species are sequestered around foreign material during the inception of a phagocytic vacuole. The reactions leading to formation of reactive oxygen species are initiated upon exposure to phagocytable particles (Klebanoff, 1980), soluble antigen-antibody complexes (Ward *et. al.*, 1983.), and various membrane perturbants and chemotactic factors (English *et. al.*, 1981; Sweeney *et. al.*, 1981). Generation of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radical is an important factor in the antimicrobial armamentarium of phagocytic cells (Nathan, *et al.*, 1983). Some organisms fail to trigger (Wilson *et al.*, 1980; Channon *et al.*, 1984; Holzer *et al.*, 1986; Eissenberg and Goldman, 1987) whereas others inhibit (Jackett *et al.*, 1978; Peasrson *et al.*, 1982; Charnetzky and Shuford, 1985; Beaman *et al.*, 1985; Buchmueller-Rouiller and Manuel, 1987; Wolf *et al.*, 1987), the production of reactive oxygen metabolites. Expression of burst activity may be affected by parasite viability (Wilson *et al.*, 1980; Locksley *et al.*, 1985), opsonization or uptake by specific macrophage surface receptors (Locksley *et al.*, 1985; Wolf *et al.*, 1987), and the state of macrophage activation (Wilson *et al.*, 1980). The production of oxygen radicals by macrophages also depends on several endogenous and exogenous factors; the site of the macrophage's residence (Drath and Karnovsky, 1975), the stage of cell maturation and, the state of macrophage activation (Fels *et al.*, 1987).



Haber-Weiss reaction:

Fig. 1: Macrophages and neutrophils are capable of generating reactive oxygen intermediates or radicals that are involved in antimicrobial activities. Those identified include superoxide anion, hydrogen peroxide, hydroxyl radicals and hypohalides. The Haber-Weiss reaction, which is proposed to be involved in generation of hydroxyl radicals, is explained separately. (Baldwin *et al.*, 1993)

1.4.2 Reactive Oxygen Species Production by Alveolar Macrophages.

Alveolar macrophages release oxygen metabolites, O_2^-, H_2O_2, and OH^- (Fantone and Ward, 1982). Among oxygen-derived reactive molecules, hypochlorous acid (HOCl) generated through the H_2O_2-myeloperoxidase system and OH^- demonstrate the highest bactericidal activity (Klebannof, 1968). In contrast to HOCl and OH^-, O_2^- has a trivial antibacterial activity, whereas H_2O_2 exhibits an intermediate activity; however, the bactericidal activity of H_2O_2 can be increased in an iron-rich environment (Quie *et al.*, 1967). Both oxidation and decarboxylation of bacterial membranes appear to be major mechanisms by which oxygen radicals induce bacterial killing (Klebanoff, 1968; Strauss *et al.*, 1970). Compared to PMN, alveolar macrophages were reported to contain little if any myeloperoxidase activity, but they may contain other peroxidases (Ross and Balm, 1980; Reynolds, 1985).

1.4.3 Reactive Oxygen Species Production by Bovine Alveolar Macrophages.

Bovine alveolar macrophages have been shown to release superoxide anion and hydrogen peroxide secondary to challenge exposure with a variety of stimuli (Dyer *et al.*, 1985; Ohmann and Babiuk, 1984). Lavage procured cells release approximately 20nmol of superoxide/h and 5 to 6 nmol of hydrogen peroxide/15 min/1,000,000 cells. These findings indicate that bovine alveolar macrophages have the oxidative pathways necessary for reactive oxygen intermediate responses.

The dominant metabolic activity displayed by bovine alveolar macrophages is the flux of glucose through the HMP shunt, regardless of either the state of bovine

alveolar macrophage stimulation or the period of lavage. Flux through the HMP shunt is threefold greater than that through the TCA cycle, and accounts for nearly 75% of the total glucose metabolized by stimulated or non-stimulated cells. Bovine alveolar macrophages metabolize small amounts of glucose for energy (Dyer *et al.*, 1989). These observations were roughly comparable with values for other species.

1.4.4 Origin of Lung Macrophages

Macrophages are part of the mononuclear phagocytic system and exist in many forms in different organs. Good evidence indicates that the bone marrow is the source of tissue macrophages, including lung macrophages. Studies in both humans and animals have shown that the majority of alveolar macrophages are derived from peripheral blood monocytes (Godleski and Brain, 1972; Blusse *et al.*, 1979; Blusse *et al.*, 1983). Although some investigators have suggested that alveolar macrophages cannot replicate within lungs, several studies demonstrate local replication of pulmonary macrophages (Reynolds *et al.*, 1976; Springmeyer *et al.*, 1982; Tarling and Coggle, 1982).

Several investigators have separated alveolar macrophages into different subpopulations (Holian *et al.*, 1983; Shellito and Kaltreider, 1984; Chandler *et al.*, 1986; Sandron *et al.*, 1986). Membrane receptor expression and cell functions, such as phagocytosis and mediator release, have been shown to vary among these different subpopulations (Shellito and Kaltreider, 1984; Chandler *et al.*, 1986; Sandron *et al.*, 1986; Hance *et al.*, 1985; Chandler and Fulmer, 1987; Sibille *et al.*, 1987).

1.5 Nitric Oxide Metabolism

1.5.1 Biological Role of Nitric Oxide

The first direct demonstration of the release of nitric oxide (NO) by mammalian cells was in the vascular endothelium where it plays a role in the control of vascular tone and platelet aggregation (Palmer *et al.*, 1987). Nitric oxide, a simple and unstable free radical, has recently been identified as a potent and pleiotropic intracellular mediator produced by and acting on many cells of the body. It acts as an endothelium-derived relaxing factor, a neuromediator and as a defence molecule of the immune system (Moncada *et al.*, 1989; Collier and Vallance, 1989). Both macrophages and neutrophils produce NO (Hibbs *et al.*, 1988).

The most exciting feature of NO is its inducibility in endothelial cells and leucocytes following stimulation with TNF and IFN-γ. When incubated with IFN-γ in combination with TNF-α, IL-1 or LPS, murine endothelial cells generate NO (Kilbourn and Belloni, 1990). TNF, interferons and endotoxin also stimulate NO production by macrophages, neutrophils, Kupffer cells and hepatocytes (Hibbs et al., 1990). A low output flux of NO modulates vascular smooth muscle and nerve action via activation of guanylate cyclase (Palmer *et al.*, 1987).

It is now clear that there are at least two distinct NO synthase isoenzymes; the constitutive enzyme responsible for basal NO synthesis in both the endothelium and the nervous system is Ca^{2+} -dependent, whereas the cytokine-inducible enzyme is Ca^{2+} -independent. This finding suggests that it may be possible to modulate immunologically-induced NO production without affecting the constitutive, house-keeping NO functions (Liew and Cox, 1991). Inducible calcium-independent NO

synthase in macrophages is produced when these cells are activated by the inflammatory cytokines IFN-γ and TNF-α. Activated macrophages have been demonstrated to metabolize the amino acid L-arginine by the oxidative pathway to produce NO/NO_2^- and citrulline (Hibbs *et al.*, 1987a,b; Marletta *et al.*, 1988; Stuehr *et al.*, 1989). NO has now been shown to be synthesized from one of the terminal guanidino-nitrogen atoms of L-arginine (Palmer *et al.*, 1988; Marletta *et al.*, 1988; Hibbs *et al.*, 1988). Nitric oxide synthase is cytosolic, NADPH-dependant and leads to the formation of stoichiometric amounts of L-citrulline and NO from L -arginine (Hibbs *et al.*, 1987a,b; Palmer and Moncada, 1989). Macrophages also produce nitrite (NO_2^-) and nitrate (NO_3^-) which are derived from L-arginine, and their production is blocked by structural analogues such as N^G -monomethyl-L-arginine (L-NMMA) (Hibbs, *et al.*, 1987a,b; Lyengar *et al.*, 1987).

It is now clear that Ca^{2+} -independent NO synthase is inducible in both the lung and liver of rats treated with endotoxin (Knowles *et al.*, 1990). Since, these organs remove infectious organisms from the blood, the host-defensive role of these organs and the involvement of NO in this process may assume considerable importance.

1.5.2 Mechanisms of NO Mediated Killing

A large flux of NO causes inhibition of iron-sulphur enzymes in target cells, blocking cell reproduction and, in some cases, inducing cell death. Reactive nitrogen intermediates have potent cytotoxic and antiproliferative effects on tumours, parasites, and bacteria (Tarr, 1941; Stuehr and Nathan, 1989; Liew and Cox, 1991).

1.5.3 Regulation of NO Synthesis by Cytokines

Up- and down-regulation of NO synthesis by cytokines, is well documented. Th-1 type cytokines (IFN-γ, IL-2) stimulate macrophages to produce NO whereas, Th-2 cytokines (IL-4, and IL-10) down-regulate NO synthesis (Liew *et al.*, 1991). Other cytokines have also been reported to exert inhibitory effects on NO synthesis by IFN-γ activated macrophages. Macrophage deactivating factor and the transforming growth factor β family block the ability of IFN-γ to induce release of reactive nitrogen intermediates from murine macrophages (Liew *et al.*, 1991; Nacy *et al.*, 1992).

1.5.4 Immunomodulatory Role of NO

The pleiotropic nature of NO function and its production by activated macrophages raises the possibility that NO also has a role as an immunoregulator (Kolb, 1992). Marcinkiewicz and Chain (1993) have reported the differential regulation of cytokine production in spleen cells by NO. It has recently been shown that suppression of allogenic or mitogenic T-cell proliferation can be mediated use of NO (Hoffmann *et al.*, 1990).

1.5.5 NO Mediated Killing of Intracellular Parasites

Nitric oxide produced from L-arginine metabolism has been reported to be involved in the killing of several intracellular parasites by mouse peritoneal macrophages (Green *et al.*, 1990; Nathan and Hibbs, 1991). Macrophages activated with IFN-γ plus LPS have a powerful cytostatic effect on the fungal pathogen *Cryptococcus neoformans* (Granger *et al.*, 1986), the protozoa *Toxoplasma gondii*

(Adams *et al.*, 1990), and *Leishmania* (Green *et al.*, 1990; Liew *et al.*, 1990). TNF-α can synergize with IFN-γ in inducing macrophage leishmanicidal activity (Bogdan *et al.*, 1990).

1.6 Host Pathogen Interaction

1.6.1 Intracellular Survival of Parasites

Once inside a professional phagocyte, pathogens face a multitude of hostile reactions. These comprise the following major mechanisms: (1) generation of reactive oxygen metabolites, (2) generation of reactive nitrogen metabolites, (3) acidification of the endosomal compartment, and (4) phagosome-lysosome fusion with subsequent attack by lysosomal enzymes and/or microbicidal peptides.

To survive inside professional phagocytes, intracellular pathogens have a variety of evasion mechanisms. In principle, these include: (1) interference with or resistance to oxidative killing, (2) inhibition of phagosome-lysosome fusion (Armstrong and Hart, 1971; Horwitz, 1983a,b), (3) interference with or resistance to lysosomal enzymes (Corrollo *et al.*, 1979) or microbicidal peptides and, (4), escape into the cytoplasm. Although the different killing and evasion mechanisms are well known phenomenologically, their relative contribution to host defence and survival in most cases remains unclear (Gendelman *et al.*, 1988).

After internalization, intracellular parasites follow one of several general pathways through the host cell. *Legionella pneumophila, Toxoplasma gondii, Chlamydia psittaci,* and *Mycobacterium tuberculosis* take an intraphagosomal route, i.e., the parasite resides in membrane bound phagosomes that do not fuse with host cell

lysosomes (Armstrong and D'Arcy Hart, 1971; Jones and Hirsch, 1972; Friis, 1972; Todd and Storz, 1975; Wyrick and Brownridge, 1978; Eissenberg *et al.*, 1983; Horwitz, 1983a,b). *Leishmania species,* including *Leishmania donovani, Leishmania mexicana,* and *Leishmania major, as well as Salmonella typhimurium and Yersinia pestis* take an intraphagolysosomal route, i.e., these parasites reside in phagosomes that fuse with lysosomes (Alexander and Vickerman, 1975; Chang and Dwyer, 1976; Chang and Dwyer, 1978; Carrol *et al.*, 1979; Berman *et al.*, 1979; Straley and Harmon, 1984). *Trypanosoma cruzi* takes an extraphagosomal route. After phagocytosis, this parasite exits from the phagosome and multiplies within the cytoplasm of the host cell (Tanowitz *et al.*, 1975; Noguiera and Cohn, 1976).

Precise identification of the molecules responsible for intracellular survival is just beginning. Over the years, sulfatides of *Mycobacterium tuberculosis*, which share a polyanionic character with substances that inhibit phagosome-lysosome fusion, and ammonia produced by *Mycobacterium tuberculosis* have been postulated to play a role in fusion inhibition by this organism. Many low molecular weight fractions of *Mycobacteria*, including sulfolipids (Pabst *et al.*, 1988; Zhang *et al.*, 1988), lipoarabinomannan (Sibley *et al.*, 1988), and phenolic glycolipid-1 (Chan *et al.*, 1989) are capable of modulating mononuclear phagocytic functions. Recently, lipoarabinomannan has been shown to scavenge cytotoxic oxygen free radicals, inhibit protein kinase C activity, and block the transcriptional activation of IFN-γ-inducible genes in human macrophage-like cell lines (Chan *et al.*, 1989; Chan *et al.*, 1991). Phenolic glycolipid-1 of *Mycobacterium leprae* and lipophosphoglycan of *Leishmania donovani* scavenge reactive oxygen metabolites in a cell free system (Neil and

Klebanoff 1988; Chan *et al.*, 1989). Glycolipid fractions from *Mycobacterium avium* serovar 2 (*Mycobacterium paratuberculosis* 18) inhibit the killing of *Candida albicans* by activated bovine peripheral-blood-derived macrophages (Hines *et al.*, 1992).

The saltatory movements of periphagosomal secondary lysosomes become slow or static in mouse peritoneal macrophages infected with live, but not with killed, *Mycobacterium microti*, and that the lysosomes of macrophages infected with live, but not killed, *Mycobacterium microti* do not fuse with a normally fusiogenic test particle (Hart *et al.*, 1987). These researchers have hypothesized that the induction of focal lysosomal stasis by *Mycobacterium microti* is the primary event resulting in inhibition of phagosome-lysosome fusion by this parasite.

The mechanisms by which *Mycobacterium avium* survives intracellularly are inadequately defined. The bacterium can inhibit superoxide anion production by macrophages (Mayer and Falkinham, 1986; Bermudez and Young, 1989) as well as phagolysosomal fusion following phagocytosis (Black *et al.*, 1990; Crowle *et al.*, 1991). It has been shown that like other mycobacteria, *Mycobacterium paratuberculosis* is phagocytized by, and replicates within, macrophages.

Phagosomes of parasites that follow the intraphagosomal route do not become acidified to the low levels typical of phagolysosomes, at least in the case of *Legionella pneumophila* (Horwitz and Maxfield, 1984) and *Toxoplasma gondii* (Sibley *et al.*, 1985). The block in acidification appears localized to the phagosome containing *Legionella pneumophila* since another phagosome in the same monocyte containing an inert particle is normally acidified (Horwitz and Maxfield, 1984). The mechanism by which these pathogens inhibit acidification is unknown.

Some studies suggest that a phenolic glycolipid (PGL-1) of *Mycobacterium leprae* may play an important role in protecting the bacterium from oxygen-dependent antimicrobial activities of mononuclear phagocytes (Neil and Klebanoff, 1988). This novel phenolic glycolipid, unique to *Mycobacterium leprae*, is synthesized by the bacterium in large amounts within the host cell (Hunter and Brennan, 1981; Hunter *et al.*, 1982). Purified PGL-1 appears to scavenge oxygen metabolites formed by macrophages (Neill and Klebanoff, 1988). Moreover, deacylated PGL-1 immunologically bound to the surface of *Staphylococcus aureus* protected this test organism from killing by non-activated and activated human monocytes (Neill and Klebanoff, 1988).

Experimental listeriosis of mice has been instrumental in analyzing the host response to intracellular bacteria (Kaufmann, 1988). Recently, the molecule, listeriolysin responsible for intracellular survival of *Listeria monocytogenes* has been identified (Mengaud *et al.*, 1988). Deletion mutants of *Listeria monocytogenes* lacking listeriolysin lost their virulence for mice and could no longer replicate in macrophage cell lines (Gaillard *et al.*, 1986; Portnoy *et al.*, 1988). This molecule promotes transition from the endosomal into the cytoplasmic compartment and is not involved in entry into host cells (Gaillard *et al.*, 1987; Kuhn *et al.*, 1988). A 60KD protein of *Listeria monocytogenes*, which seems to be responsible for entry, has been identified (Kuhn and Goebel, 1989). *Shigella flexneri* may escape phagosomes by a similar process (Sansonetti *et al.*, 1986; Clerc *et al.*, 1987).

1.7 Activation of Macrophages by Cytokines

1.7.1 Macrophage Activation by Interferon-γ (IFN-γ)

IFN-γ has been shown to enhance macrophage antimicrobial (Murray and Cohn, 1980; Nathan *et al.*, 1980) and oxidative (Klebanoff, 1982; Ito and Krim, 1985) activities. IFN-γ has been shown to activate human monocytes in such a way that they kill or inhibit the multiplication of *Legionella pneumophila* (Bhardwai *et al.*, 1986; Nash *et al.*, 1988), *Toxoplasma gondii* (Nathan, *et al.*, 1983; Nathan and Tsunawaki, 1984; Murray *et al.*, 1985; Wilson and Westfall, 1985), *Chlamydia psittaci* (Rothermel *et al.*, 1983), *Leishmania donovani* (Murray *et al.*, 1983; Nacy *et al.*, 1983; Hoover *et al.*, 1985), *Leishmania major* (Passwell *et al.*, 1986), and *Trypanosoma cruzi* (Reed *et al.*, 1987).

Macrophages exposed to recombinant IFN-γ (rIFN-γ) develop the ability to secrete reactive oxygen intermediates, as well as to kill nonspecifically obligate or facultative intracellular microorganisms (Murray *et al.*, 1985; Flesch and Kaufmann, 1987). Similar observations have been reported for the activation of cultured human monocytes (Nathan *et al.*, 1983). Van Dissel *et al.*, (1987) concluded that, as a single activating stimulus, rIFN-γ is not capable of activating the antibacterial effector function of peritoneal macrophages against facultative intracellular pathogens, such as *Listeria monocytogenes* and *Salmonella typhimurium*. Treatment of human monocytes with IFN-γ decreased phagocytosis and had no effect on the intracellular replication of *Mycobacterium avium*, suggesting that IFN-γ does not have macrophage activating activity for *Mycobacterium avium*-infected human monocytes (Tsuyuguchi *et al.*, 1990). Using agents that modulates phagosome lysosome fusion, Flesch and Kaufmann

(1987) have suggested that phagosome lysosome fusion is involved in the growth inhibition of *Mycobacterium bovis* by rIFN-γ activated macrophages. Growth inhibition of *Mycobacterium bovis* by IFN-γ activated macrophages is an oxygen-independent process (Flesch and Kaufmann, 1988).

The mechanisms by which IFN-γ activated phagocytes exert an antimicrobial effect on intracellular parasites are not completely understood. Some mechanisms, however, have been proposed: (1) IFN-γ induced degradation of tryptophan has been proposed as the mechanism by which activated fibroblasts and epithelial cells inhibit the replication of *Toxoplasma gondii* and *Chlamydia psittaci*, whose growth is dependent upon the availability of this amino acid (Pfefferkorn *et al.*, 1986; Byrne, *et al.*, 1986), (2) IFN-γ induced limitation of iron availability has been proposed as the mechanism by which human monocytes inhibit the multiplication of *Legionella pneumophila* (Byrd and Horwitz, 1987). In support of this hypothesis, it has been demonstrated that *Legionella pneumophila* intracellular multiplication is iron-dependent and that the capacity of IFN-γ activated monocytes to inhibit *Legionella pneumophila* intracellular multiplication is completely neutralized by the addition of iron (Byrd and Horwitz, 1987).

1.7.2 Macrophage Activation by Tumor Necrosis Factor-α (TNF-α)

It has been demonstrated that TNF can stimulate bacteriostatic and bactericidal activities in macrophages (Bermudez *et al.*, 1992). TNF appears to participate in the immune response against *Mycobacterium avium* (Bermudez and Young, 1988; Bermudez *et al.*, 1989), *Mycobacterium tuberculosis* (Kaufmann, 1989; Rook *et al.*,

1989), *Chlamydia trachomatis* (Williams *et al.*, 1990), *Listeria monocytogenes* (Nakane, et al. 1988; Desiderio et al.1989), *Candida albicans* (Djeu, *et al.*, 1986), *Legionella pneumophila* (Blanchard *et al.*, 1988). It has been proposed that IFN-γ produced by T cells activates macrophages to secrete TNF which, in turn, sensitizes the same or other macrophages to secrete ROS that rapidly destroy parasites through a process of lipid peroxidation (Clark *et al.*, 1986). Treatment with TNF-α or GM-CSF led to restriction of mycobacterial growth in macrophages (Denis, 1991). A virulent strain of *Mycobacterium* (Erdman) differs from an attenuated strain of *Mycobacterium* (H37Ra) in that lipoarabinomannan it contains mannose oligosaccharide "caps" on the arabinose saccharides at the terminal portions of the molecule. This difference can influence TNF secretion from murine macrophages (Chatterjee *et al.*, 1992a,b).

1.7.3 Macrophage Activation by Other Cytokines

Interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), and macrophage colony stimulating factor (M-CSF) activate human monocyte/macrophage antimicrobial activity against several intracellular pathogens, including *Trypanosoma cruzi* (Reed *et al.*, 1987) *Leishmania donovani* (Weiser *et al.*, 1987) *Leishmania amazonensis* (Ho *et al.*, 1992; Ho *et al.*, 1990), and *Mycobacterium avium* complex (Bermudez *et al.*, 1989; Bermudez and Young, 1989; Bermudez and Young, 1990).

The potent antimicrobial nature of migration inhibitory factor (MIF) has been demonstrated by the inhibition of growth of a panel of isolates of *Mycobacterium avium* in cultures of bone-marrow-derived murine macrophages and human monocytes.

These cells, when cultured in MIF, exhibit various signs of activation, including cell division, morphological changes such as evidence of substantial phagolysosomal fusion, and enhanced secretion of TNF (Orme *et al.*, 1992). In this latter regard, it is intriguing to recollect that the first observed action of MIF was control of monocyte migration (Bloom and Bennet, 1966; David, 1966), presumably a critical factor in the recruitment of monocytes into sites of mycobacterial infection and subsequent granuloma formation.

1.8 Regulatory Role of Macrophages

Macrophages have been shown to promote or modulate lymphocyte activity. Macrophages support T-cell mediated immune responses by antigen processing and presentation and by secretion of soluble mediators (Rosenthal and Shevach 1973; Unanue and Allen, 1987); macrophages also may suppress lymphocyte proliferation by releasing hydrogen peroxide, prostaglandins, NO and other suppressive mediators (Kung *et al.*, 1977; Allison, 1978; Metzger, 1980; Isobe and Nakashima, 1992). Prostaglandins have long been known to be key effector molecules in mediating inflammatory reactions, and have more recently been shown to regulate the production of several T-cell-derived cytokines selectively (Betz and Fox, 1991; Marcinkiewicz *et al.*, 1992). Suppression of allogenic or mitogenic T-cell proliferation can be mediated by NO (Hoffmann *et al.*, 1990).

1.8.1 Genetic Control of Resistance and Susceptibility to Infection

It has been demonstrated that Ity, Bcg, and Lsh, (all assumed to be the same

gene or a closely linked complex) influence the net rate of multiplication of *Salmonella typhimurium*, *Mycobacterium bovis*, strain BCG, *Leishmania donovani*, and *Mycobacterium lepraemurium* within macrophages of the mononuclear phagocytic system (Plant and Glynn, 1976; Bradley *et al.*, 1979; Hormaeche, 1980; O'Brien *et al.*, 1980; Skamene *et al.*, 1982; Brown *et al.*, 1982).

Studies with transposon mutants of *Salmonella typhimurium* revealed that numerous genes must be involved in resistance to macrophage killing and, hence, underline the complexity of the factors related to intracellular survival (Fields *et al.*, 1986; Buchmeier and Heffron, 1989). This strategy has recently led to the identification of a gene which encodes resistance to defensins and possibly to other antimicrobial mechanisms of professional phagocytes (Fields, 1989).

There is evidence for genetic control of resistance and susceptibility to infections with *Mycobacterium lepraemurium* in inbred mice, with the *Ity (Lsh, Bcg)* gene controlling resistance to infections via the intravenous route (Brown *et al.*, 1982), and many genetic variables controlling resistance and susceptibility to cutaneous inoculation (Closs and Haugen, 1975). It now appears that the *Ity (Lsh, Bcg)* gene is expressed at the level of the mature macrophage, although the precise mechanism responsible for preventing bacterial growth in *Ity^r (Lsh^r, Bcg^r)* cells is still unclear (Denis *et al.*, 1990).

Numerous substances are known to modify macrophage function. Immune modulators can induce phagocytic recruitment and activity, enhance phagocytosis, and increase the phagocytes' capacity to destroy pathogen (McCollough and Martinoid, 1987). Tuftsin is a naturally occurring biologic tetrapeptide known to activate many

recognize macrophage and granulocyte functions: phagocytosis and pinocytosis, motility, chemotaxis, and bactericidal and tumoricidal activities (Najjar *et al.*, 1983). Addition of tuftsin enhanced the ability of the permissive macrophages to control the intracellular replication of *B. abortus* and resulted in the functional conversion of the permissive macrophages into restrictive macrophages (Price *et al.*, 1993).

2. OBJECTIVES

Haemophilus somnus is a Gram-negative, small, fastidious, bacterium that has been recognized as a facultative intracellular pathogen of cattle (Corstvet *et al.*, 1973; Crandell *et al.*, 1977; Humphrey *et al.*, 1982; Humphrey and Stephens, 1983; Lederer *et al.*, 1987; Harris and Janzen, 1989; Corbeil, 1990). It causes a vide variety of clinical syndromes in cattle collectively known as haemophilosis. *H. somnus* is able to interact with a number of cells types in the host, including phagocytes. *In vitro* studies have established that bovine PMN are unable to kill *H. somnus*, and that *H. somnus* can replicate within bovine monocytes (Lederer *et al.*, 1987). Additional studies demonstrate that bovine alveolar macrophages and blood monocytes ingest but do not kill opsonized *H. somnus* (Lederer *et al.*, 1986). These findings suggest that the bacterium can persist and proliferate within these cells and contribute to the pathogenesis of haemophilosis.

The objective of this study was to investigate the interaction between bovine mononuclear phagocytes and *H. somnus*. The first specific objective was to investigate the effect of *H. somnus* on phagocytic function of bovine mononuclear phagocytes (BMP). The second specific objective was to characterize the intracellular survival kinetics of *H. somnus* in freshly isolated and various recombinant bovine cytokine treated BMP. The third specific objective was to investigate the bactericidal ability of BMP following interaction with *H. somnus*. The fourth specific objective was to investigate the endogenous TNF, IFN-γ, and IL-6 response to *H. somnus* infection.

3. BACTERIUM-HOST CELL INTERACTIONS: FLUORESCENT LABELLING OF *HAEMOPHILUS SOMNUS* AND ANALYSIS OF BACTERIUM-PHAGOCYTE INTERACTION BY FLOW CYTOMETRY

ABSTRACT

The interactions between bovine mononuclear cells and *Haemophilus somnus* are known to be complex. To study this interaction, a flow cytometric assay was developed to assess the effect of *H. somnus* on phagocytosis of opsonized *S. aureus* by bovine alveolar macrophages and bovine blood monocytes. Using this *in vitro* system, it was found that logarithmically growing *H. somnus* significantly inhibited the phagocytosis of opsonized *S. aureus* by bovine alveolar macrophages obtained both from healthy calves and from cattle experimentally infected with *H. somnus*. However, neither heat- or formalin-killed log-phase *H. somnus* nor, *in vitro* passaged *H. somnus* showed any effect on the phagocytic activity of these cells. In contrast to bovine alveolar macrophages, bovine blood monocytes showed a significant increase in their phagocytic activity following *in vitro* exposure to logarithmically growing *H.somnus*. These results suggest that the ability of *H. somnus* to modulate bovine mononuclear phagocytic function may contribute towards the pathogenesis of the disease.

INTRODUCTION

Phagocytic cells are responsible for ingesting and destroying bacterial pathogens. Pathogenic bacteria have developed a variety of mechanisms for evading destruction by host defences (Densen and Mandell, 1980). The ability to survive and multiply within cells of the mononuclear phagocytic system is a strategy that has been utilized by a number of important pathogens of humans and domestic animals, including Mycobacteria (Lurie, 1942), *Brucella* (Smith and Fitzgeorge, 1964), *Salmonella* (Furness, 1958), *Yersinia* (Cavanaugh and Randall, 1959), and *Listeria* (Mackaness, 1964) species. By surviving inside mononuclear phagocytes, these microbes are protected from the immune system and are able to cause local and systemic infections.

Haemophilus somnus is a bovine pathogen that causes significant economic loss in the beef and dairy cattle industries (Kennedy *et al.*, 1960; Corstvet *et al.*, 1973; Stephens *et al.*, 1981). *H. somnus* can enter a variety of organs and cause several disease syndromes including thrombotic meningoencephalitis (Kennedy *et al.*, 1960; Bailie *et al.*, 1973; MacDonaled *et al.*, 1973; Humphrey, 1982; Blood *et al.*, 1983; Little, 19986; Harris and Janzen, 1989), septicemia (Humphrey, 1982; Blood *et al.*, 1983; Little, 1986; Harland *et al.*, 1990), myocarditis (Humphrey, 1982; Harris and Janzen, 1989; Harland *et al.*, 1990), arthritis (Humphrey, 1982; Harris and Janzen, 1989; Harland *et al.*, 1990), pneumonia (Crandell *et al.*, 1977; Humphrey, 1982; Andrews *et al.*, 1985; Corbeil *et al.*, 1986; Little, 1986; Gogolewski *et al.*, 1987a; Bryson, *et al.*, 1990), pleuritis (Humphrey, 1982; Blood *et al.*, 1983; Little, 1986; Harland *et al.*, 1990), abortions (Humphrey, 1982; Corbeil *et al.*, 1986; Corbeil *et al.*,

1987; Harris and Janzen, 1989), and synovitis and conjunctivitis (Humphrey, 1982; Blood *et al.*, 1983; Harris and Janzen, 1989; Barnum, 1990).

Since it has been reported that *H. somnus* is able to survive and multiply in bovine alveolar macrophages and blood monocytes (Lederer *et al.*, 1987) *H. somnus* is classified as a facultative intracellular pathogen. Moreover, it has been reported that bovine neutrophils (PMN) are unable to kill ingested *H. somnus* (Czuprynski and Hamilton, 1985).

Flow cytometry provides a potentially powerful tool for analyzing bacterium-host cell interactions, particularly if fluorophores which do not affect the viability of the bacteria or macrophages are used (Raybourne and Bunning, 1994). Although florescent antibodies have been used to identify intracellular, attached, and freely suspended bacteria, this approach may alter the viability or receptor properties of bacteria that are labelled before exposure to eucaryotic cells (Donnelly and Baiegant, 1986; Buchmeier and Heffron, 1989). Moreover, fluorescent-antibody binding is dependent on the availability of epitope(s) and the stability of the antigen-antibody reaction. Direct labelling of bacteria with fluorescein derivatives can affect protein moieties on the bacterial surface; leaching of the dye is also a major concern. Nucleic acid-binding dyes, chromomycin A3, and acridine orange have been used in flow cytometric analysis (Van Dilla *et al.*, 1983; Miliotis, 1991), but these dyes are toxic, making bacterial recovery impossible.

The lipophilic dye PKH2 (Sigma Immuno Chemicals, St. Louis, MO), which has been used to label mammalian cell membranes and bacteria, is stable, nontoxic, and does not affect the functional properties of the cells (Horan and Slezak, 1989;

Horan *et al.*, 1990; Raybourne and Bunning, 1994). In this study, parameters have been established for labelling both log and stationary phase *H. somnus* with this fluorophore and a flow cytometric assay has been used to study the interactions between bovine mononuclear phagocytes and *H. somnus.*

MATERIALS AND METHODS

Bacteria

A pathogenic strain of *H. somnus* (HS25) originally isolated from the pneumonic lung of a calf was obtained from Alberta Agriculture in Edmonton, Alberta was maintained in egg yolk, at -70C. Egg yolk stock culture was made by inoculating the pure cultures into 6-7 day old embryonated chicken eggs and incubating them at 37C until death of embryo occurred 1-3 days later. The egg yolks were then removed under sterile conditions, aliquoted, and stored at -70C. The isolate HS25 was selected for this study based on past research at V.I.D.O., including studies of interaction between PMN and *H. somnus* (Pfeifer, 1992), and bovine animal model studies (Harland *et al.*, 1990). Before each experiment, an aliquot of egg yolk stock culture was thawed and spread onto a 5% sheep blood agar plate (PML Microbiologicals, Richmond, B.C.) and incubated for 36 h at 37C in an atmosphere containing 5% CO_2. A loopful of colonies was transferred to BHITT [Brain Heart Infusion (Difco, Detroit, MI) supplemented with 1 µg/ml of 1% thiamine monophosphate (Sigma, St. Louis, MO), and 10 µg/ml of 10% Trizma base (Sigma, St. Louis, MO)], and incubated aerobically for 10 h at 37C on a shaker. As the final step, this broth culture was diluted to 1:4 with BHITT and incubated for 2 h until the optical density (OD) (660

nm) reached 0.4. One ml of this broth was found to contain approximately $1X10^9$ logarithmically growing *H. somnus*. Stationary phase *H. somnus* cultures were aerobically grown for 24 h at 37C in 10 ml of BHITT on a shaker. One ml of this broth contained approximately $1X10^9$ stationary phase *H. somnus*. The actual number of bacteria per ml was determined by plating 10-fold serial dilutions of the suspension in duplicate and counting colonies. This number was used to calculate the ratio of phagocytes to bacteria in all experiments. *H. somnus* was recovered and washed twice with cold (4C) Hanks balanced salt solution (HBSS) by centrifugation before being used. Both logarithmically growing and stationary phase *H. somnus* were killed by treating with 0.1% formalin for 18 h at 37C or by heating at 65C for 30 min. Killed *H. somnus* were resuspended in calcium and magnesium-free phosphate buffered saline (PBSAG) and stored at -20C. *In vitro* passaged *H. somnus* were prepared by passaging egg yolk stock cultures for 12-15 passages on sheep blood agar plates before producing logarithmically growing *H. somnus*.

The culture of *Staphylococcus aureus* used was originally isolated from a case of bovine mastitis and was subsequently frozen at -70C in skim milk as a pure stock culture. A sample of stock culture was thawed and spread on a 5% sheep blood agar plate and incubated for 24 h at 37C in an atmosphere containing 5% CO_2. After incubation, a colony of *S. aureus* was selected, and incubated in 100 ml of Luria-Bertani (LB) broth (Difco, Detroit, MI) that was grown aerobically for 18 h at 37C on a shaker. One ml of this seed culture was mixed with 100 ml of LB-Broth and incubated for 6 h at 37C on a shaker. Approximately $1X10^9$ CFU of *S. aureus* were in 1 ml of this broth.

Source and Isolation of Bovine Mononuclear Phagocytes

Bovine alveolar macrophages (BAM) and bovine blood monocytes (BBM) were obtained from clinically normal, 2-3 month old dairy and 6-12 month old beef calves. These animals were maintained at V.I.D.O. research facilities at the University of Saskatchewan. Heparinized (Organon Teknika, Toronto, Canada) blood was centrifuged for 5 min at 37C and 1000 g to separate the buffy coat from the platelet rich plasma. The buffy coat was removed and diluted with HBSS and layered on Ficoll-Paque (Pharmacia LKB, Biotechnology, Uppsala, Sweden) to obtain a mononuclear cell-rich population. The cells from the interface were removed and washed twice with HBSS. Isolated peripheral blood mononuclear cells (PBMC) were suspended in macrophage SFM-medium (serum free medium) with glutamine (Gibco BRL, Life Technologies Inc. Grand Island, NY) supplemented with 10% fetal bovine serum (heat inactivated) (Gibco BRL, Life Technologies Inc. Grand Island, NY). Mononuclear cell viability was determined by trypan blue exclusion, and averaged 95%. PBMC suspensions contained between 4-10% monocytes as determined by microscopic examination of stained smears (Diff-Quick Fixative, Baxter Healthcare Corporation, IL).

Isolation of Bovine Alveolar Macrophages

Lung lavages were performed using an endotracheal tube on anesthetized calves. Three hundred ml of warm (37C) HBSS solution were used for each lavage. Lavage fluid was filtered with a nylon strainer after recovery and centrifuged at 4C for 20 min at 800 g. Cell pellets were suspended in HBSS and layered on to Ficoll-Paque to obtain mononuclear cells. Mononuclear cells were removed in the interphase,

washed twice with HBSS and suspended in macrophage medium. Alveolar macrophage viability, determined by trypan blue exclusion, averaged 95%. Lung cell suspensions contained between 65%-85% macrophages as determined by microscopic examination of stained smears (Diff-Quick Fixative, Baxter Healthcare Corporation, IL).

The *H. somnus* Bovine Experimental Model

A field isolate of *H. somnus* (HS25) was injected intravenously into 6-8 month old beef calves (Harland *et al.*, 1990; Schuh *et al.*, 1991). A challenge dose contained approximately $5X10^7$ colony forming units (CFU). Clinical signs occurring after challenge included fever, depression, and polyarthritis. The challenge was fatal to 60%-70% of the calves and the necropsy findings included meningitis, encephalitis (Gomis *et al.*, 1993), myocarditis, pericarditis, pneumonia, pleuritis and polyarthritis. This bovine *H. somnus* model closely reproduced the clinical and pathological features of the disease as seen in Western Canada.

For the FC assay, blood samples were taken from these experimentally infected animals on day 1, 6, 7, 9, and 12 post-infection and as well as calves of the same age and breed housed in similar conditions. Lung lavages were performed after euthanasia of moribund animals to obtain BAM.

PKH2 Labelling of *H. somnus*

Five ml of logarithmically growing or stationary phase *H. somnus* culture were centrifuged, washed once in PBS, and resuspended in 0.5 ml of labelling buffer (Sigma, St. Louis, MO). From this suspension, 50 µl was taken and mixed with 0.5 ml

of labelling buffer in a polypropylene tube (Becton Dickinson, NJ). PKH2 dye (7.5 µl) was mixed with 0.5 ml of labelling buffer in another polypropylene tube. The contents of two tubes were mixed and incubated at room temperature for 10 min in the dark; the reaction was then stopped by adding 10 ml of phosphate buffered saline containing 0.5% bovine serum albumin (PBS-BSA). The labelled bacteria were washed twice with 10 ml of PBS-BSA and resuspended in PBS-BSA. The actual number of bacteria per ml was determined by plating 10-fold serial dilutions of the suspension in duplicate. This number was used to calculate the ratio of phagocytes to bacteria in all experiments. Further, the effect of labelling with PKH2 on the viability of *H. somnus* was determined by counting CFU of labelled and unlabelled bacteria by plating 10-fold serial dilutions of the suspension in duplicate. The fluorescent intensity of log and stationary phase *H. somnus* was measured by flow cytometer before the beginning of each infection experiment to ensure that the labelling procedure was adequate.

PI- Labelling and Opsonization of *S. aureus*

Propidium iodide (PI) (Sigma, St. Louis, MO) labelling of *S. aureus* was carried out according to the method of Pfeifer *et al.*, (1992). Briefly, heat killed *S. aureus* were mixed with 0.5% PI solution and incubated at room temperature for 30 min in the dark, and then washed four times with normal saline. This PI-labelled *S. aureus* solution was adjusted to OD (620 nm) of 2.5 in PBSAG (phosphate buffered saline with glucose and gelatin), and then aliquoted and stored at -70C.

Anti-*Staphylococcus aureus* bovine convalescent sera came from animals that had recovered from *S. aureus* mastitis. *S. aureus* was opsonized using anti-*S. aureus*

bovine convalescent serum. Fifty µl of stock *S. aureus* and 50 µl of serum were mixed and incubated for 30 min at 37C in a shaking water bath. The bacteria and sera were separated by centrifugation, the serum removed and the bacteria resuspended in 1 ml of PBSAg.

Flow Cytometric (FC) Phagocytosis Assay

This assay was conducted in 96 well microtitre plates (Corning Glass Works, NY). To each microtitre well, 70 µl of opsonized *S. aureus* (red fluorescence), 20 µl of PKH2-labelled unopsonized *H. somnus* (green fluorescence) and 100 µl of isolated PBMC or BAM were added and mixed by pipetting. PBSAG was used for the controls. Plates were then incubated for 1 h at 37C with gentle mixing. After incubation, 20 µl of 300 mM ethylenediamine-tetraacetic acid (EDTA) (J.T. Baker Chemical Co. Phillipsburg, NJ) were added to each well and plates were centrifuged at 400 g for 4 min. The supernatant was discarded and cell pellets were washed twice with 200 µl of PBSAG. After washing, 200 µl of lysostaphin (Sigma, St. Louis, MO) (44 U/ml) were added to each well, and incubated for 30 min at 37C in an atmosphere containing 5% CO_2 to destroy any non-phagocytized *S. aureus* remaining on cell surfaces. At the end of 30 min, the plates were centrifuged as in the above step to remove the supernatant, and then cell pellets were washed twice with PBSAG. As the final step, plates were placed on ice for 1 min before resuspending the cells in PBSAg containing 2% formalin. Samples were filtered using a nylon filter before reading them by the flow cytometer. Cell populations for the FC assay were selected by gating the desired population based on their size (forward angle light scatter) and granularity (90° light

scatter) (Haynes, 1988; Shapiro, 1988; Sucic *et al.*, 1989). Bitmaps were drawn to select monocytes or alveolar macrophages (Fig. 1) and 5000 monocytes or alveolar macrophages were routinely examined for each sample. In addition to the size and granularity of mononuclear cells as the selection criteria, a monocyte/granulocyte specific marker (DH59 monoclonal antibody conjugated with fluorescein isothiocyanate) was used to determine more accurately the gating (data not shown). Fluorescent microscopic examination of BBM and BAM demonstrated phagocytosis of *S. aureus* and *H. somnus* (Fig. 2). Light with a wavelength of 488 nm was used to excite the fluorescent molecules. Red fluorescence from PI was collected through a 585/42 nm band absorbance filter. Green fluorescence from PKH2 was collected through a 530/30 nm band absorbance filter in combination with a 560 nm dichroic mirror. Phagocytosis of PI-labelled *S. aureus* and PKH2-labelled *H. somnus* was analyzed further using the Lysis II (Becton Dickinson, Canada, Inc. Mississauga, ON) and Reproman ver. 1.6 computer programs (Reproman, True Facts Software Inc., Seattle, WA). A quadrant structure was used to distinguish positive from negative cells (Fig.3). Quadrant 1 represented BBM or BAM that had actively phagocytized PI-labelled *S. aureus*; quadrant 2 represented BBM or BAM that had actively phagocytized both *S. aureus* and *H. somnus*; quadrant 3 represented non-active or 'non-responder' BBM or BAM; quadrant 4 represented BBM or BAL that had phagocytized PKH2-labelled *H. somnus* but not PI-labelled *S. aureus*. All samples were analyzed using fluorescence compensation to omit the overlap of fluorescence seen between PI and PKH2 emissions (Shapiro, 1988; Hasui *et al.*, 1989).

Flow cytometric studies were done using the Becton Dickinson FACScan flow

cytometer. (Becton Dickinson, Canada, Inc. Mississauga, ON) with a 15 MW argon laser light source. Diagnostic checks on the FC machine were performed daily using CaliBrite flowcytometer beads (Becton Dickinson, Canada, Inc. Mississauga, ON). Propidium iodide fluorescence was determined using a Spectro Fluorometer Model 430 (G.K. Turner & Associates).

To examine the effect of soluble products of *H. somnus* in contrast to contact with *H. somnus*, a 0.4 μm membrane containing transwell plates (Costar, Cambridge, MA.) was used to separate *H. somnus* from phagocytes (Pfeifer, 1992).

Recombinant Bovine Cytokines

Recombinant bovine IL-1β (rBoIL-1β) (American Cyanamid Co., Princeton, NJ) was produced in an *E. coli* expression system. The stock solution of 6.8 mg of rBoIL-1β/ml had a specific activity of $7.7X10^6$ IU/mg protein as determined by a murine thymocyte proliferation assay. There was 125 EU of endotoxin/mg of protein as determined by the limulus amoebocyte lysate assay (Whittacker Corp., Walkersville, MD). The recombinant bovine granulocyte-macrophage colony stimulating factor (rBoGM-CSF) (American Cyanamid Co., Princeton, NJ) was produced in an *E. coli* expression system. Recombinant BoGM-CSF had a concentration of 2.7 mg/ml with an endotoxin content of 30.2 EU/mg of protein, and specific activity of $11.8X10^6$ U/mg (50% proliferation of neonatal bovine bone marrow). Recombinant bovine IFN-γ (rBoIFN-γ) (Ciba-Geigy, Basel, Switzerland) had a protein concentration of 0.54 mg/ml, > 95% purity, and specific activity was $3.0X10^6$ U/mg protein. The endotoxin content was 1.0 ng/mg protein. Recombinant bovine TNF-α (rBoTNF-α) (Genentech

Inc., San Francisco, CA) was produced in an *E. coli* expression system. Recombinant BoTNF-α had a protein concentration of 0.4 mg/ml, and specific activity was $1X10^{(6)}$ U/mg protein as determined by cytotoxicity of L929 cells. For the FC assay, 100 ng/ml of recombinant bovine cytokines and 5 μg/ml of *E. coli* LPS (serotype 0111:B4) (Sigma Chemical Co. St. Louis, MO) were used to activate BBM and BAM. Both BBM and BAM were incubated for 2 h including 1 h during the phagocytosis assay with these recombinant cytokines and *E. coli* LPS.

Statistical Analysis

Analysis of variance (ANOVA) was executed using Minitab (Minitab for Windows, ver 9.2, Minitab Inc., 3081, Enterprise Drive, State College, PA.) in a Randomized Complete Block Design, to test the significance of the treatments. Statistical significance was assumed at $P \leq 0.05$. Mean comparisons were carried out using least significant difference (Lsd) test whenever the F test for treatment was significant at the 5% level.

RESULTS

FC Analysis of Phagocytosis of Opsonized *S. aureus* by BAM and BBM

Bovine mononuclear cells were gated according to cell size and granularity in order to differentiate alveolar macrophages or monocytes from lymphocytes (Fig. 1). A quadrant system was used for analysis of red fluorescence for actively phagocytizing cells versus 'non-responders'. Quadrant 1 represented cells that were actively phagocytizing *S. aureus*. Quadrant 3 represented cells that were not active and

remained fluorescently negative (Fig. 3 (A) & (B)).

The presence of live, logarithmically growing *H. somnus* decreased phagocytosis of opsonized *S. aureus* by BAM (21 ± 1.58 %) compared to their normal phagocytosis (52.4 ± 3.36 %) ($P<0.0001$) when the phagocyte to bacteria ratio was 1:100 (Fig. 4 & 5). Phagocytosis by BAM was not decreased when either heat or formalin killed log phase *H. somnus* or *in vitro* passaged *H. somnus* were used (data not shown). The inhibitory effect was not seen when *H. somnus* and BAM were partitioned by a 0.4 μm pore size membrane. Stationary phase *H. somnus* increased the phagocytosis of opsonized *S. aureus* compared to normal levels (71.2 ± 15.42 %, 52.4 ± 3.36 %, respectively; $P<0.031$) (Fig. 6).

In contrast to BAM, phagocytosis of *S. aureus* by BBM was increased both by logarithmically growing and stationary phase *H. somnus*. There was no decrease in phagocytosis of *S. aureus* by BBM even with a high ratio of bacteria to phagocytes (Fig. 7 & 8).

Since *in vitro* addition of *H. somnus* modulated the function of bovine mononuclear phagocytic cells from healthy calves, the effect of *H. somnus* in animals with the clinical disease was studies. For this study, BAM and BBM were obtained from experimentally infected cattle. Since this study was conducted to compare *in vitro* with *ex vivo*, observations no *in vitro* addition of *H. somnus* was made. The observations were similar with regard to BBM of both experimentally infected animals and clinically normal animals (Table 1). However, there was no decrease in phagocytosis of opsonized *S. aureus* by BAM of experimentally infected animals as compared to *in vitro* studies with logarithmically growing *H. somnus* (data not shown).

Effect of *H. somnus* on Phagocytes

Fluorescent intensity was closely related between logarithmically growing and stationary phase *H. somnus* (Fig. 9). Because of the observation of an inhibitory effect on phagocytosis by BAM caused by log-phase *H. somnus*, the FC assay was modified by using PKH2-labelled *H. somnus* to determine the actual uptake of *H. somnus*. The PKH2 labelling did not have any effect on viability of either logarithmically growing or stationary phase *H. somnus*.

Figure 10, panel B, demonstrates the effect of logarithmically growing *H. somnus* on BAM. *H. somnus* was able to enter BAM while selectively inhibiting *S. aureus* uptake. As shown in Fig. 10 panel C, stationary phase *H. somnus* entered BAM without any effect on *S. aureus* uptake. Similarly, both logarithmically growing and stationary phase *H. somnus* were able to enter BBM without any inhibitory effect on *S. aureus* uptake (data not shown). In fact, *S. aureus* uptake by BBM was slightly increased, as illustrated in Figure 4. Opsonisation of *H. somnus* with hyperimmune serum did not enhance phagocytosis.

Effect of Cytokine Activated BBM and BAM

The kinetics of phagocytosis of rBo-IFN-γ, rBo-TNF-α, rBo-GM-CSF, rBo-IL-1β, and *E. coli* LPS treated BAM and BBM were similar to those with untreated cells. There was no noticeable difference in the uptake of opsonized *S. aureus* by BBM or BAM between cytokine treated and cytokine untreated cells.

DISCUSSION

A FC assay using fluorescent labelled bacteria was developed to detect phagocyte-bacterium interactions, which are important in the development of immunity and the ability of a pathogen to survive in the host (Fields *et al.*, 1986; Berche *et al.*, 1987; Brunt *et al.*, 1990; Bancroft *et al.*, 1991). In this FC assay, PI-labelled opsonized *S. aureus* was used as an indicator of phagocytosis. The labelling of live *H. somnus* with PKH2 was stable and nontoxic. The viability of labelled and unlabelled *H. somnus* was indistinguishable. The methodology used in this FC assay was a refinement of previously described reports (Hasui *et al.*, 1989; Pfeifer *et al.*, 1992). For the FC assay described here, isolated monocytes or alveolar macrophages were used instead of whole blood or unpurified cells because *H. somnus* can survive in bovine PMN, in addition to their survival and multiplication in bovine mononuclear phagocytes (Czuprynski and Hamilton, 1985; Lederer *et al.*, 1987). Using isolated mononuclear cells instead of unpurified cells, it was possible to overcome possible competition between PMN and mononuclear phagocytes for *H. somnus*. Advantages of this FC assay system included simultaneous measurement of four cellular parameters (cell size, cell granularity, and phagocytosis of PI-labelled *S. aureus* and PKH2-labelled *H. somnus*) and the ability to process a large number of cells in a short period of time while examining each of the cells individually. The timing of the assay tended to be restrictive, as the samples had to be read on the flow cytometer within 6 h of being processed. If left for more than 24 h (at 4C, in the dark) the non-responder cells became more highly fluorescent (red fluorescent). The time restriction for reading of PKH2-labelled *H. somnus* by flow cytometer was not determined.

These experiments demonstrated that *H. somnus* was able to modulate the phagocytic function of BBM and BAM. Log-phase *H. somnus* caused the inhibition of *S. aureus* uptake by BAM. Moreover, by using the PKH2-labelled *H. somnus*, it was possible to rule out non-specific inhibition of phagocytic functions of BAM. Further, a moderate stimulation of *S. aureus* uptake by BAM was seen as a result of interaction with stationary phase *H. somnus*. The inhibition of *S. aureus* uptake by BAM was not due to competition by *H. somnus* for *S. aureus* receptors on the BAM since formalin- or heat-killed, *in vitro* passaged, and stationary phase *H. somnus* did not cause any decrease in phagocytosis as seen with log-phase *H. somnus*, and exposure to the other forms of *H. somnus* actually tended to stimulate *S. aureus* uptake. There are two possibilities to explain the inhibitory effect on *S. aureus* uptake by BAM: first, there may be a secreted product of log-phase *H. somnus* that mediated this action; or second, phagocytized bacteria may mediate an inhibitory effect on BAM. Using a membrane to separate *H. somnus* from BAM, it was possible to rule out a secretory product-mediated inhibition. Therefore, it appeared that a component of live, logarithmically growing *H. somnus* was required when they were inside BAM for the suppression of *S. aureus* uptake. This observation is particularly relevant when one considers that bovine mononuclear phagocytes are the cell type within which *H. somnus* is able to multiply (Lederer *et al.*, 1987). Moreover, the ability of unopsonized *H. somnus* to enter bovine mononuclear phagocytes, and their ability to multiply within phagocytes would, in turn, protect them from other host defences, such as neutralizing antibodies and other opsonins, as well as from previously activated phagocytes. Furthermore, the ability of *H. somnus* to survive and multiply within bovine mononuclear phagocytes,

could in turn, assist the bacterium to invade tissues and help to transport it across cell barriers and, thus, disseminate the disease.

Recombinant bovine cytokine or *E. coli* LPS treatment did not modulate the phagocytic function of BBM or BAM; this might be associated with insufficient duration of incubation of mononuclear phagocytes with these cytokines.

Phagocytic function of BBM from both experimentally infected animals and healthy animals demonstrated similar results with *H. somnus*. The selective inhibitory effect on *S. aureus* uptake by BAM was not obvious in *ex vivo* experiments unless log-phase *H. somnus* were added *in vitro* to the assay (data not shown). Factors that might contribute to the differences observed between *ex vivo* and *in vitro* results may include: differences in the length of time that BAM were exposed to *H. somnus;* the ratio of bacteria to BAM within the animal; the potential unavailability of infected BAM to the assay due to the procedure of lung lavage; different cytokine mediated effects. The ability of *H. somnus* to interfere with phagocytic function of BBM and BAM may contribute to its ability to survive and multiply within bovine mononuclear phagocytes. The results of these *in vitro* and *ex vivo* experiments indicated the ability of *H. somnus* to modulate the phagocytic functions of BBM and BAM. This cellular modulation might be important in the pathogenesis of haemophilosis.

Fig. 1: Bitmaps or gating of cell populations within peripheral blood mononuclear cells for the FC assay. The X axis represents the cell size (forward angle light scatter), and the Y axis represents the cell granularity (90° light scatter). Cells in the area 1 are mononuclear phagocytes. The cells out side area 1 are mainly lymphocytes.

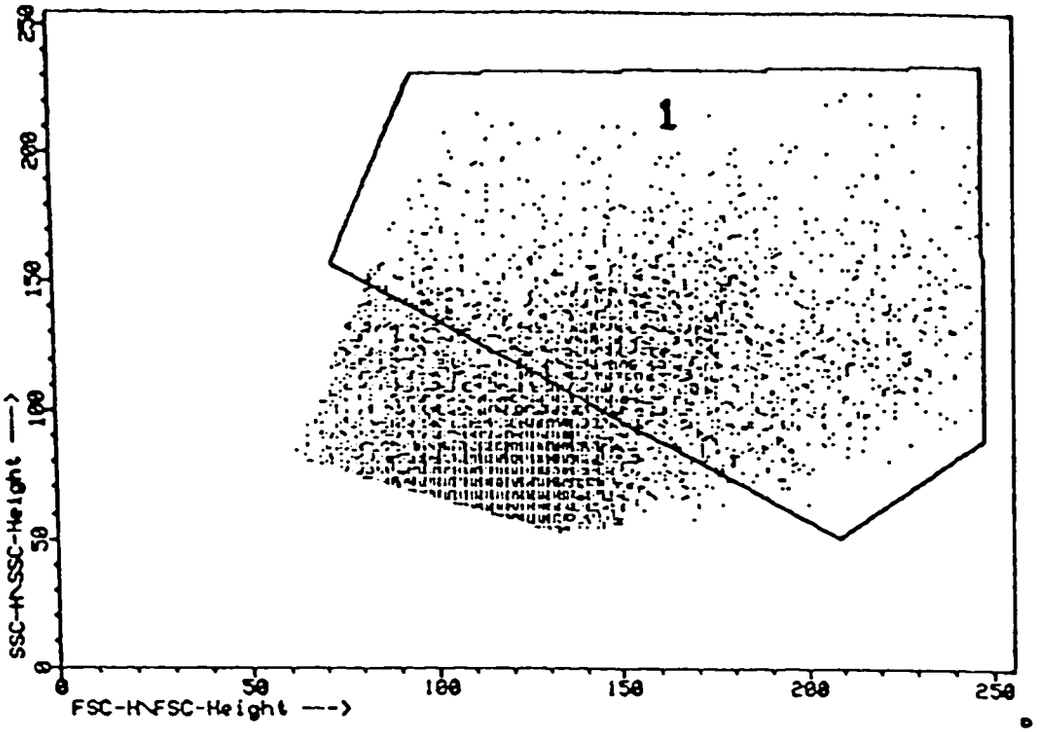

1
SSC-H\SSC-Height —>
FSC-H\FSC-Height —>
0
50
100
150
200
250

Fig. 2: Photomicrographs of alveolar macrophages containing fluorescent labelled bacteria. (A) PI-labelled *S. aureus* (red fluorescence) in alveolar macrophages. (B) PKH2-labelled *H. somnus* (green fluorescence) and PI-labelled *S. aureus* (yellow fluorescence) in alveolar macrophages. (The red fluorescence changed to yellow fluorescence when colour filters were adjusted for green fluorescence in the fluorescent microscope.)

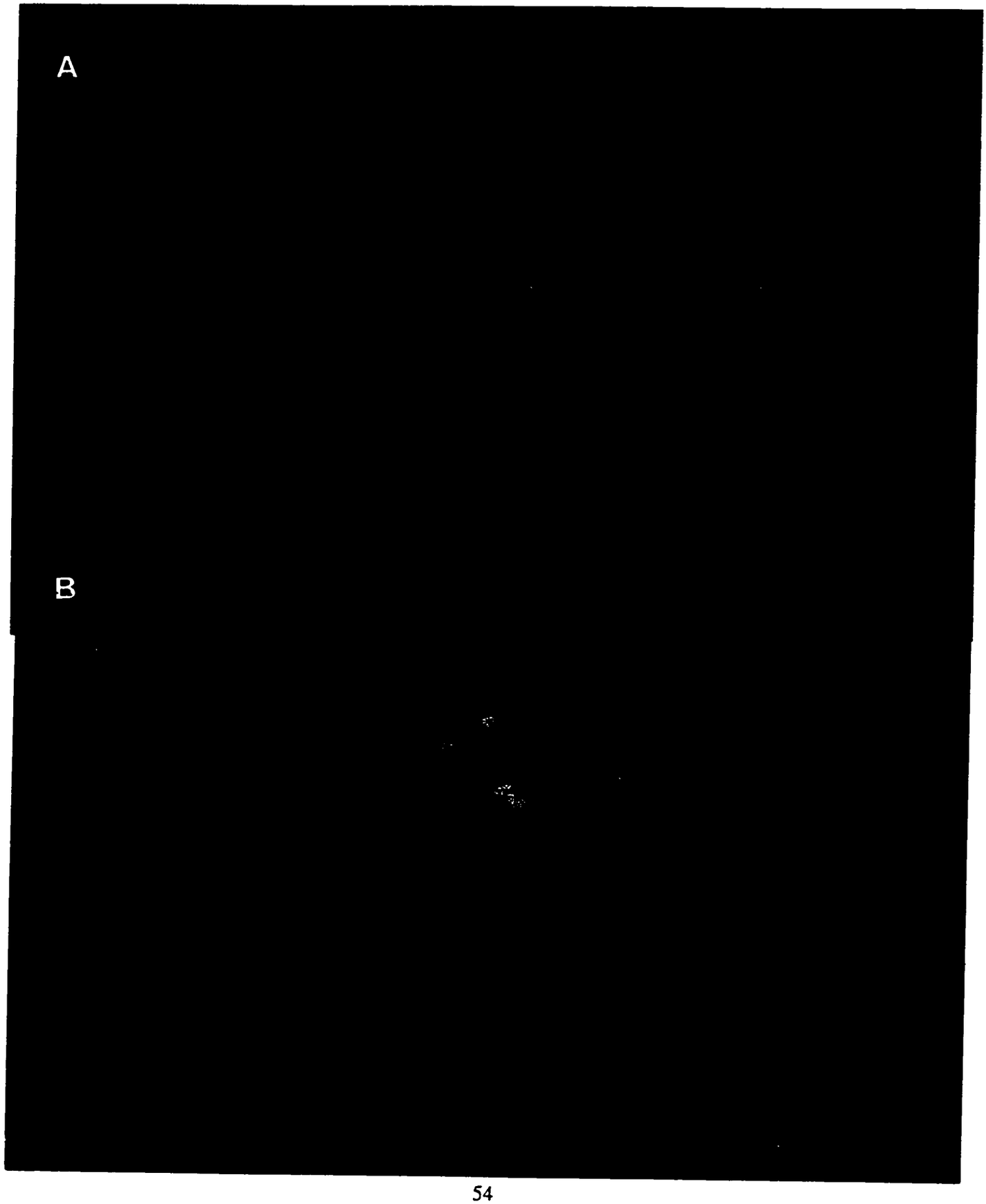

A
B

Fig. 3: Quadrant analysis of histograms of bovine alveolar macrophages (BAM) using PI-labelled *S. aureus* (red fluorescence) and PKH2-labelled *H. somnus* (green fluorescence). Quadrant 1 contains only PI-labelled *S. aureus*. Quadrant 2 contains both PI-labelled *S. aureus* and PKH2-labelled *H. somnus*. Quadrant 3 contains non-active or 'non-responder' cells. Quadrant 4 contains only PKH2-labelled *H. somnus*. The X axis represents the red fluorescence, and the Y axis represents green fluorescence (A) Control sample containing BAM without addition of labelled bacteria. BAM remain in quadrant 3 as 'non-responders'. (B) BAM containing PI-labelled *S. aureus*; actively phagocytizing BAM in quadrant 1 and 'non responder' BAM in quadrant 3. (C) BAM containing PKH2-labelled *H. somnus*; actively phagocytizing BAM in quadrant 4 and 'non-responder' BAM in quadrant 3. (D) BAM containing both PI-labelled *S. aureus* and PKH2-labelled *H. somnus*; BAM containing only *H. somnus* remain in quadrant 4 while BAM containing both *S. aureus and H. somnus* remain in quadrant 2.

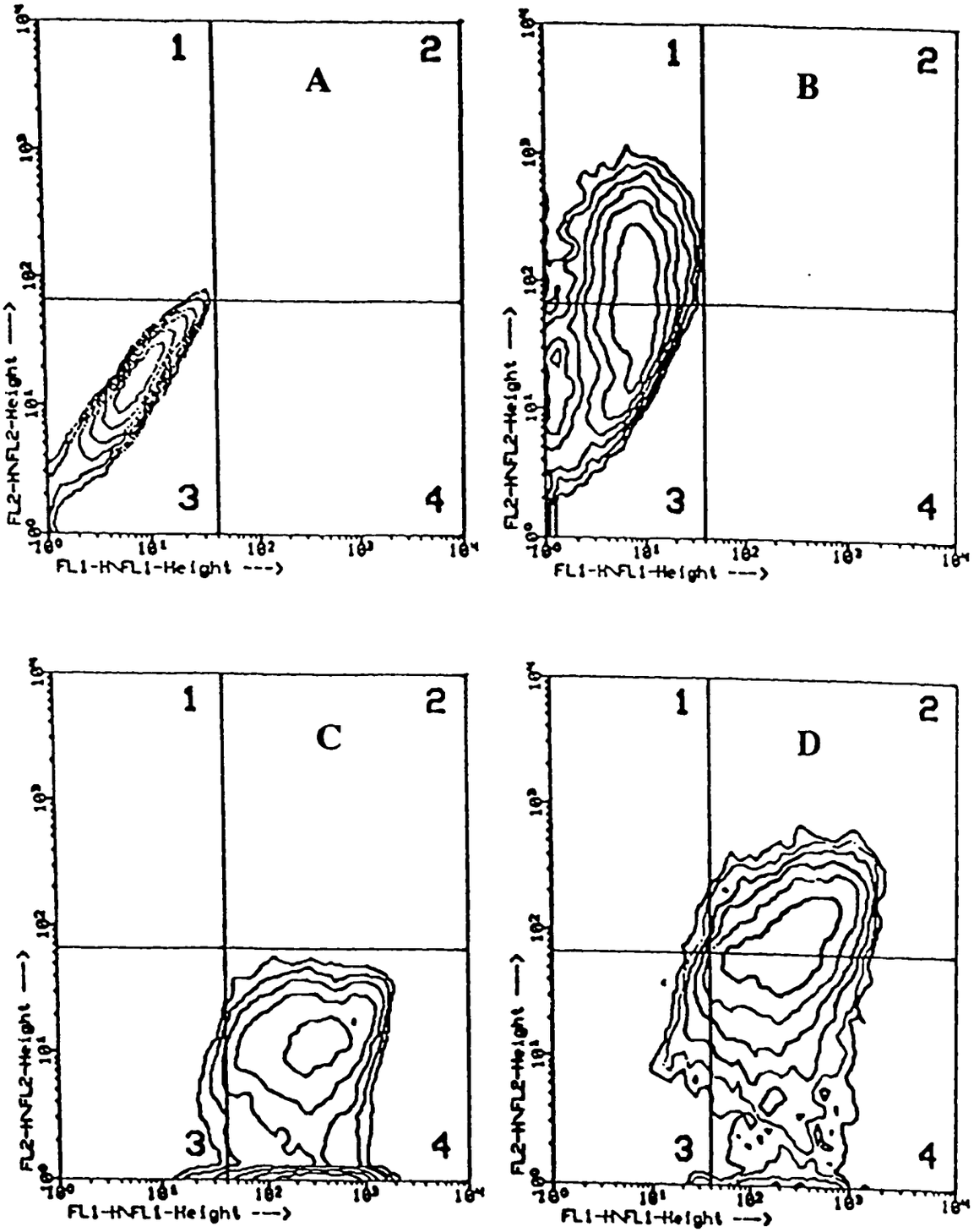
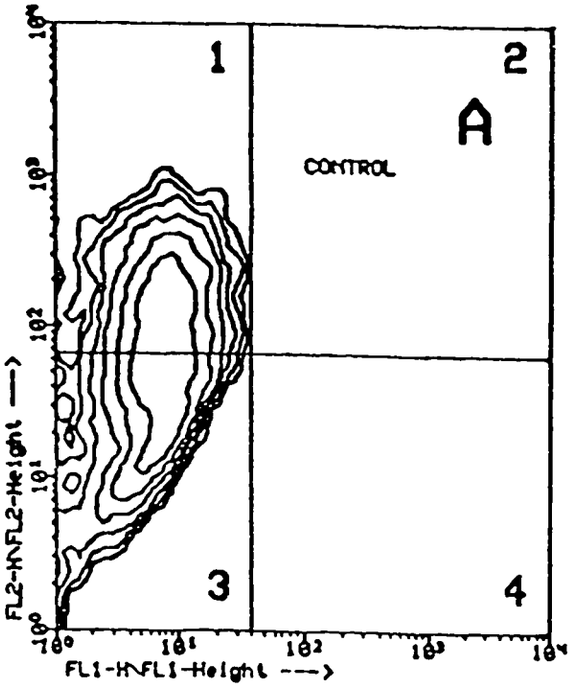
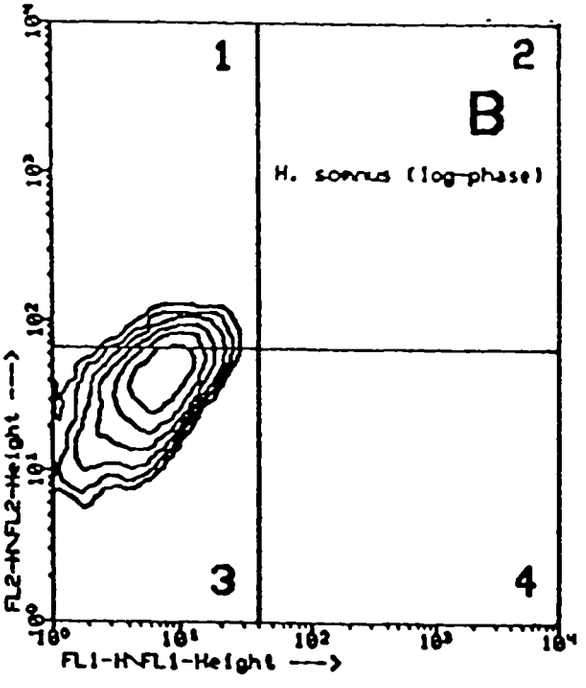

A
B
C
D
1
2
3
4
FL2-HNFL2-Height --->
FL1-HNFL1-Height --->

Fig. 4: The effect of logarithmically growing and stationary phase *H. somnus* on bovine alveolar macrophages (BAM) as shown by FC assay using PI-labelled *S. aureus* to monitor phagocytic function. (A) A BAM sample, with quadrant 1 containing 50% of actively phagocytizing alveolar macrophages. (B) A BAM sample treated with log-phase *H. somnus*, with quadrant 1 containing 13% of actively phagocytizing alveolar macrophages. (C) A BAM sample treated with stationary-phase *H. somnus*, with quadrant 1 containing 56% of actively phagocytizing alveolar macrophages.


1
2
A
CONTROL
3
4
FL2-H\FL2-Height ---->
FL1-H\FL1-Height --->


1
2
B
H. somnus (log-phase)
3
4
FL2-H\FL2-Height ---->
FL1-H\FL1-Height ---->

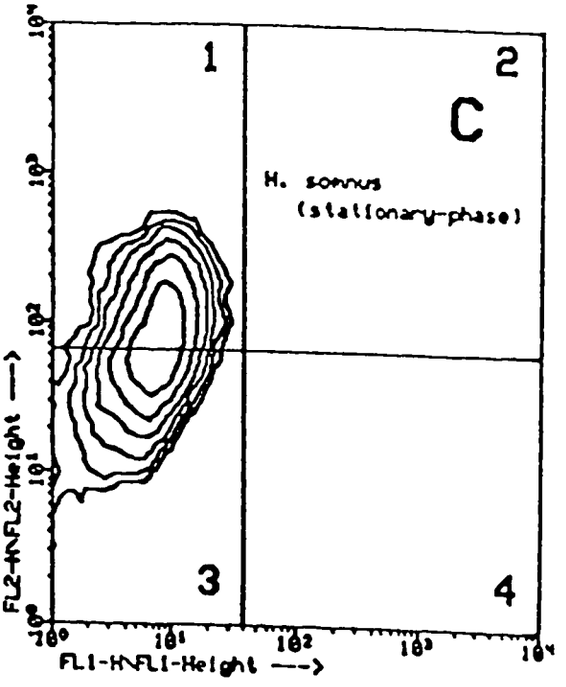

1
2
C
H. somnus
(stationary-phase)
3
4
FL2-H\FL2-Height ---->
FL1-H\FL1-Height ---->

Fig. 5: Effect of logarithmically growing *H. somnus* on phagocytosis of *S. aureus* by bovine alveolar macrophages (BAM). Control bar represents phagocytosis of *S. aureus* alone (52.4 ± 3.36 %). There was a significant decrease in phagocytic function of BAM by log-phase *H. somnus* (21 ± 1.58 %), when the phagocyte to bacteria ratio was 1:100 ($P<0.0001$).

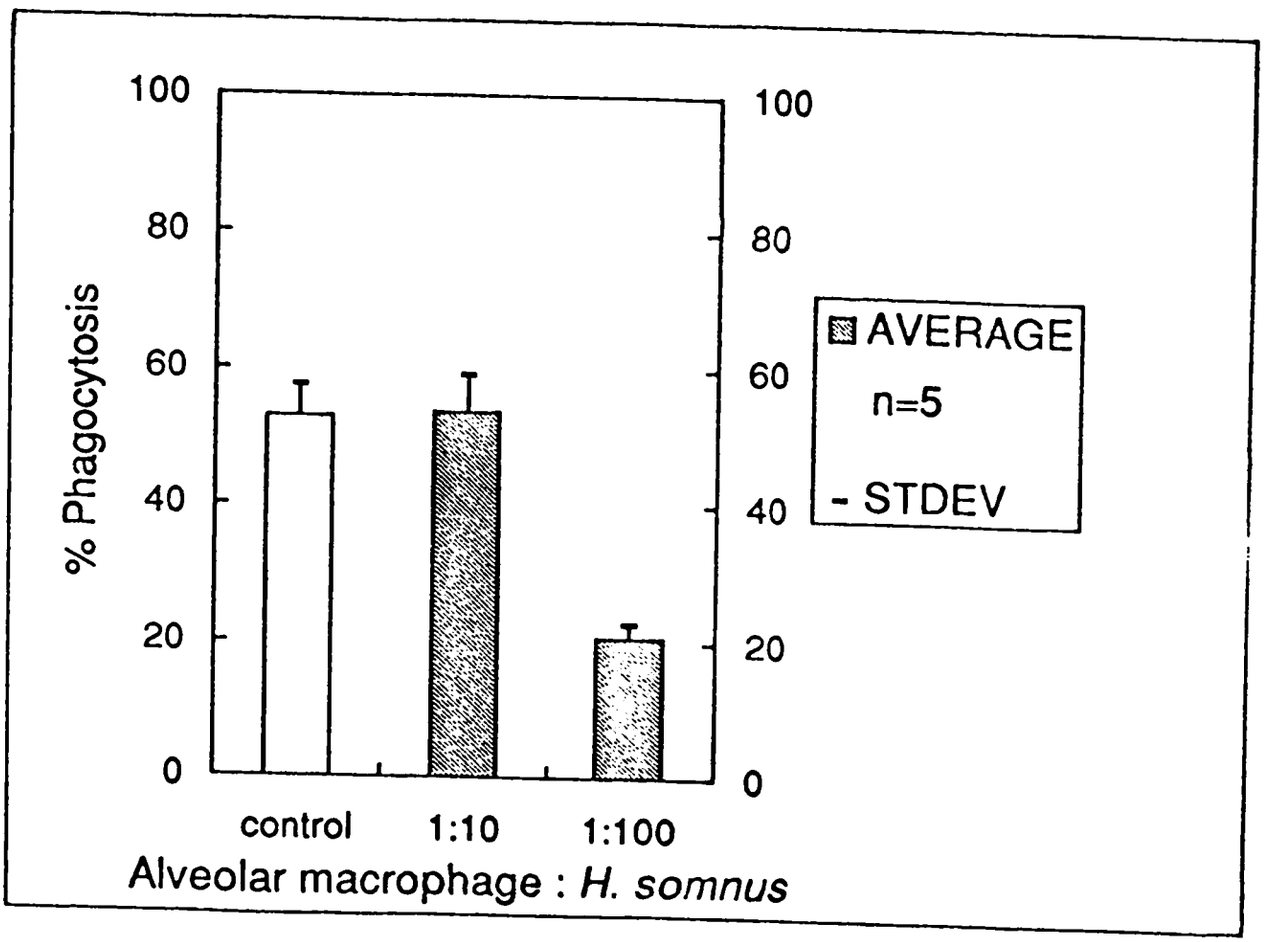

100
80
60
40
20
0
% Phagocytosis
control
1:10
1:100
AVERAGE
n=5
- STDEV
Alveolar macrophage : H. somnus

Fig. 6: Effect of stationary phase *H. somnus* on phagocytosis of *S. aureus* by bovine alveolar macrophages (BAM). There was a increase of phagocytic function of BAM, when the ratio of phagocyte to bacteria ratio was 1:100 (52.4 ± 3.36 %) (71.2 ± 15.42 %) ($P<0.031$).

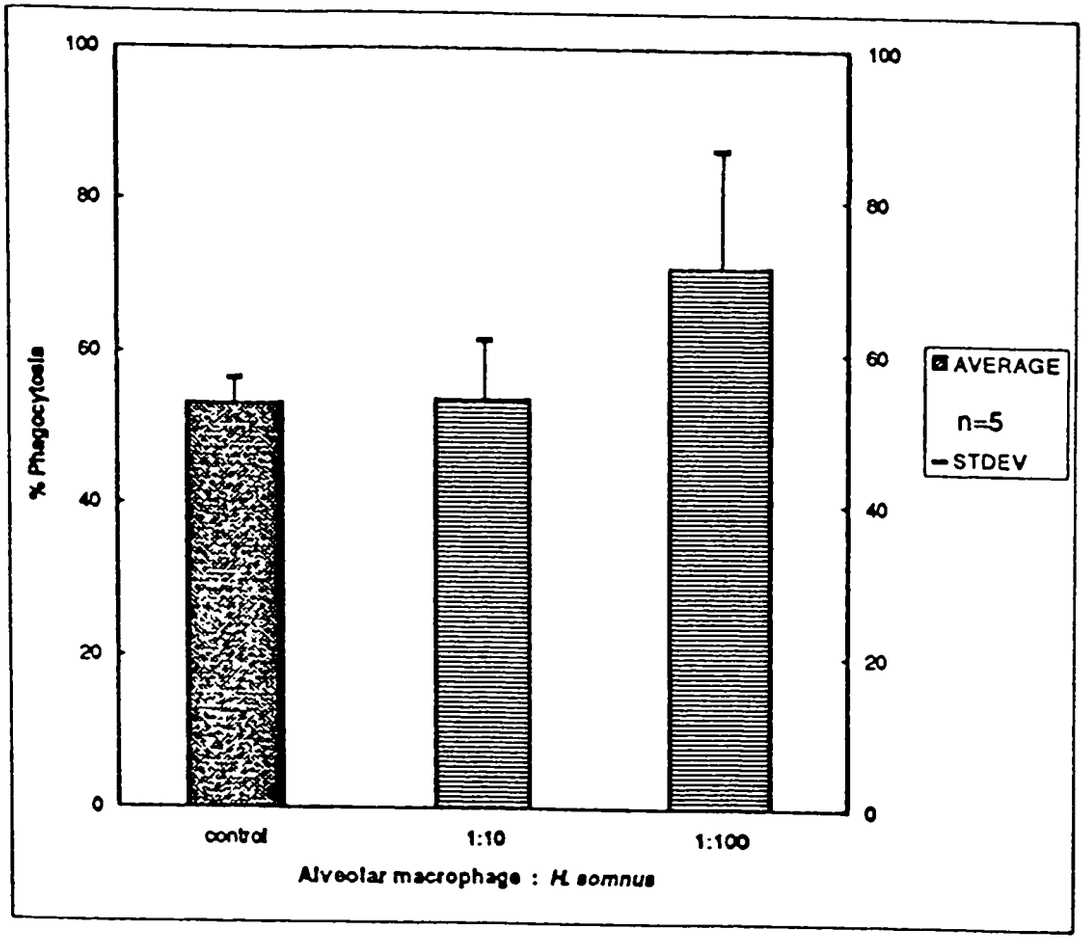

100
80
60
40
20
0
% Phagocytosis
control
1:10
1:100
Alveolar macrophage : H. somnus
AVERAGE
n=5
STDEV

Fig. 7: Effect of logarithmically growing *H. somnus* on phagocytosis of *S. aureus* by bovine blood monocytes (BBM). There was a gradual increase in phagocytic function of BBM with increased number of log-phase *H. somnus*.

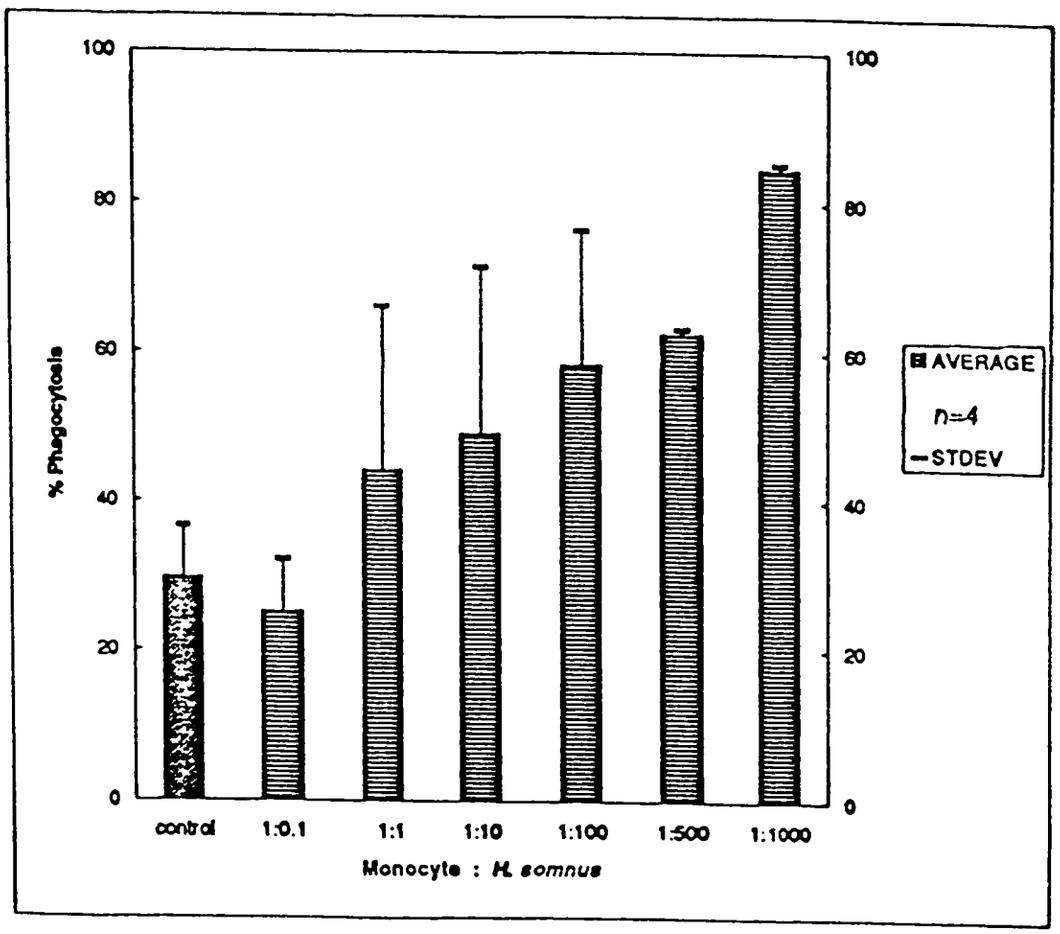

100
80
60
40
20
0
% Phagocytosis
control
1:0.1
1:1
1:10
1:100
1:500
1:1000
Monocyte : H. somnus
AVERAGE
n=4
STDEV

Fig. 8: Effect of stationary-phase *H. somnus* on phagocytosis of *S. aureus* by bovine blood monocytes (BBM). There was a gradual increase in phagocytic function of BBM with increased number of stationary phase *H. somnus.*

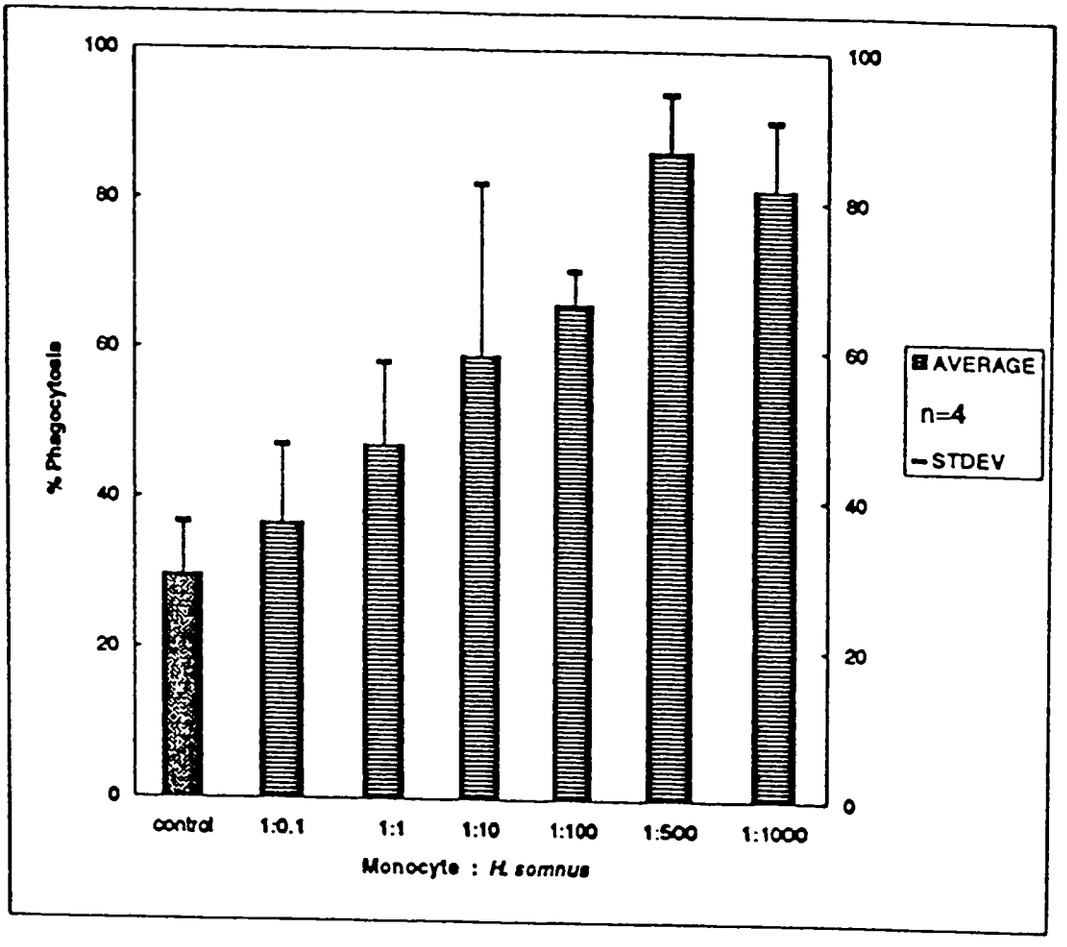
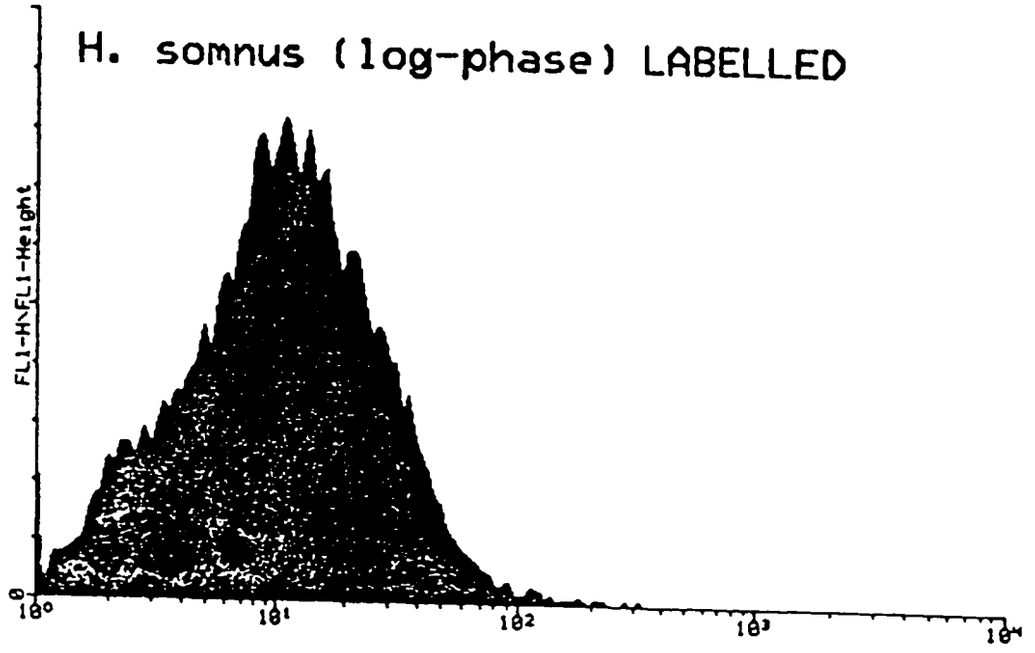

100
80
60
40
20
0
% Phagocytosis
AVERAGE
n=4
STDEV
control
1:0.1
1:1
1:10
1:100
1:500
1:1000
Monocyte : H. somnus

Fig 9: Linear green fluorescence histograms of logarithmically growing *H. somnus* and stationary-phase *H. somnus* immediately after labelling with PKH2.


H. somnus (log-phase) LABELLED
FL1-H\FL1-Height
0
10⁰
10¹
10²
10³
10⁴

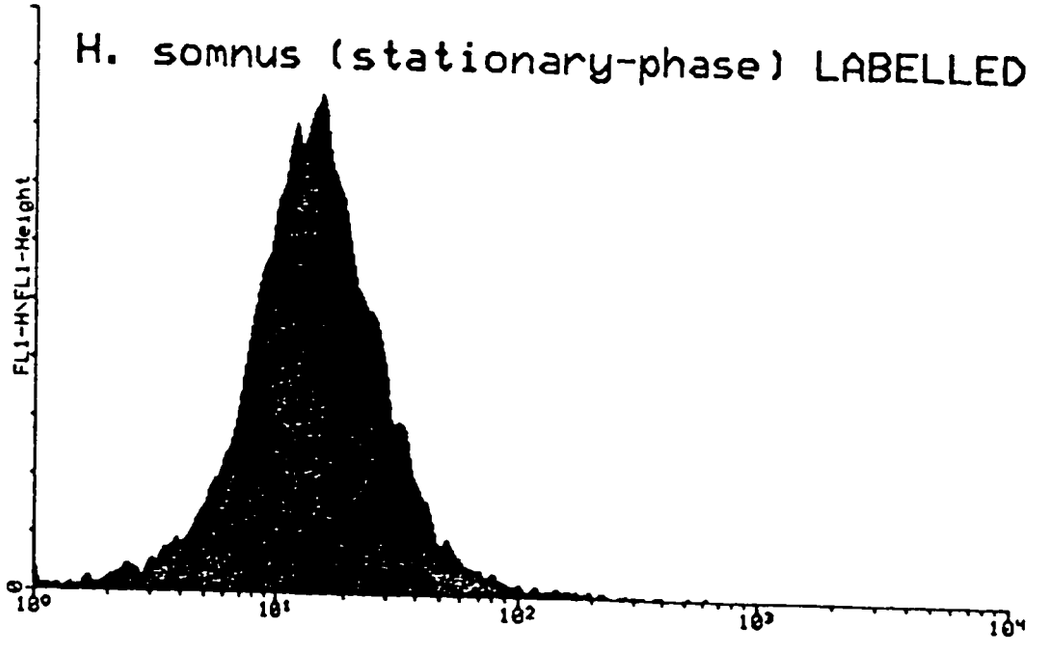
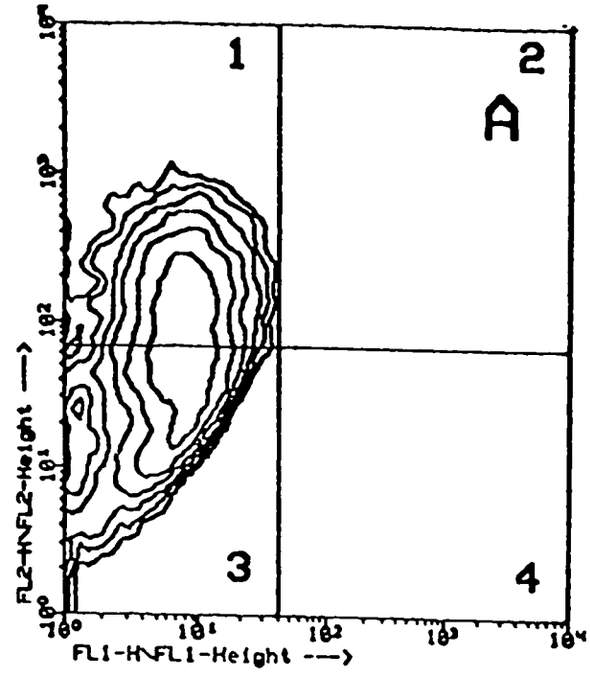
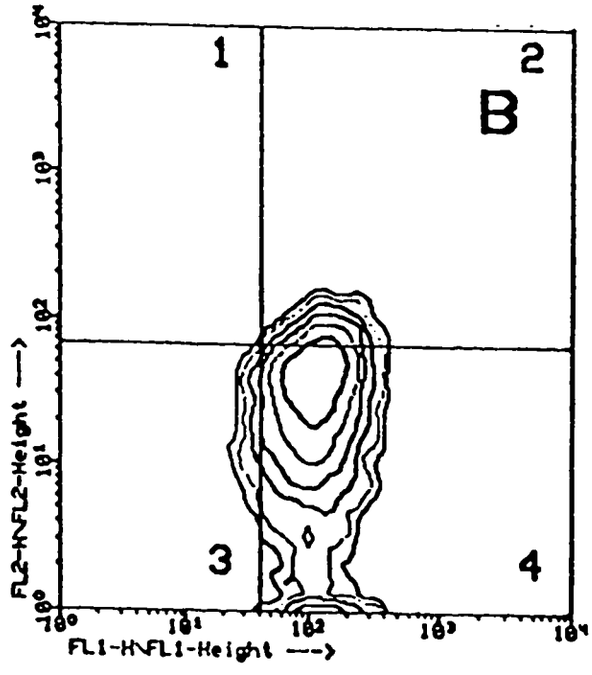

H. somnus (stationary-phase) LABELLED
FL1-H\FL1-Height
0
10⁰
10¹
10²
10³
10⁴

Fig. 10: Effect of log-phase and stationary phase *H. somnus* on bovine alveolar macrophages (BAM). Analysis of FC assay data with dual colour fluorescence. (A) Phagocytosis of PI labelled *S. aureus* by BAM: quadrant 1 (52%) and 3 (47%) represent PI-labelled *S. aureus* in the control sample. (B) Effect of log-phase *H. somnus* on phagocytosis of PI labelled *S. aureus:* quadrant 2 (12% of both *S. aureus* and *H. somnus*) and 4 (85% of *H. somnus*) represented selective inhibition of *S. aureus* uptake *by* BAM. (C) Effect of stationary-phase *H. somnus* on phagocytosis of PI labelled *S. aureus*: quadrant 2 (50% of both *S. aureus* and *H. somnus*) and 4 (46% of *H. somnus*) represented phagocytic function of BAM with stationary phase *H. somnus*. Histogram (A), Y axis represented red fluorescence. Histograms (B) and (C); Y axis represented red fluorescence and X axis represented green fluorescence.


1
2
A
3
4
FL2-H\FL2-Height ---->
FL1-H\FL1-Height --->


1
2
B
3
4
FL2-H\FL2-Height ---->
FL1-H\FL1-Height ---->

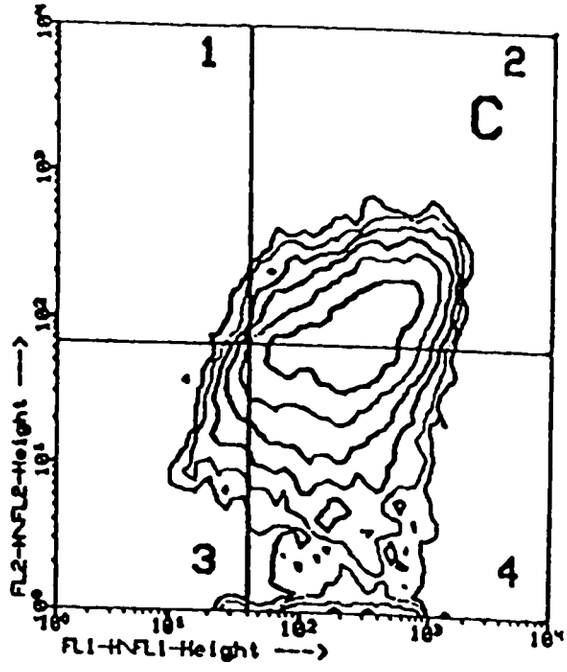

1
2
C
3
4
FL2-H\FL2-Height ---->
FL1-H\FL1-Height ---->

Table 1: A comparison of phagocytosis of opsonized *S. aureus* by bovine blood monocytes (BBM) between healthy animals and cattle experimentally infected with *H. somnus*. FC assay was conducted in experimentally infected animals and control animals in the same day to prevent day to day variation. The flow cytometric phagocytosis assay was conducted on BBM on day 1, 6, 7, 9, and 12 post-infection. Note a higher percentage of phagocytosis in some days post-infection with *H. somnus* (marked with *). The higher % of phagocytic function of BBM in some experimentally infected animals could be associated with intracellular *H. somnus*; this possibility is discussed in chapter 4. [con=control animals, Inf=Infected animals, Inf3 died on day 8 post-infection with *H. somnus*]

Table 1: A comparison of phagocytosis of opsonized *S. aureus* by BBM between healthy animals and cattle experimentally infected with *H. somnus*.

	% Phagocytosis						
DAY	Con1	Con2	Con3	Inf1	Inf2	Inf3	Inf4
1	14	17	26	17	63*	22	
6	20	34	22	34	26	14	
7	17	23	23	29	41	23	
9	36	41	42	52*	37		45
12	31	34	25	44	35		55*

4. INTRACELLULAR SURVIVAL OF *HAEMOPHILUS SOMNUS* IN BOVINE BLOOD MONOCYTES AND ALVEOLAR MACROPHAGES

ABSTRACT

The mechanisms used by *Haemophilus somnus* to survive and multiply within bovine mononuclear phagocytes are not fully understood. In order to study the interaction between bovine mononuclear phagocytes and *H. somnus*, a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was developed to assess the survival of *H. somnus* within cultured bovine blood monocytes (BBM). Using this system, it was found that: (1) *H somnus* was able to survive within BBM *in vitro* and the kinetics of its survival were similar to that seen in BBM isolated from experimentally infected cattle; (2) treatment of BBM with rBoIFN-γ, rBoTNF-α, rBoIL-1β, or rBoGM-CSF had no significant effect on the survival of *H. somnus*. Moreover, using ultrastructural studies, and ^{3}H uracil incorporation into nucleic acids (^{3}H uracil assay), it was possible to demonstrate the survival of *H. somnus* in freshly isolated bovine mononuclear phagocytes. These results indicate that the ability of *H. somnus* to survive and multiply in both freshly isolated and cytokine treated cultured BBM could be important in pathogenesis of bovine haemophilosis.

INTRODUCTION

Haemophilus somnus is a Gram-negative, pleomorphic bacterium responsible for a variety of disease manifestations in cattle. *H. somnus* can enter a variety of organs and cause diseases including thrombotic meningoencephalitis (Kennedy *et al.*, 1960; Bailie *et al.*, 1973; MacDonaled *et al.*, 1973; Humphrey, 1982; Blood *et al.*, 1983; Little, 19986; Harris and Janzen, 1989), septicemia (Humphrey, 1982; Blood *et al.*, 1983; Little, 1986; Harland *et al.*, 1990), myocarditis (Humphrey, 1982; Harris and Janzen, 1989; Harland *et al.*, 1990), arthritis (Humphrey, 1982; Harris and Janzen, 1989; Harland *et al.*, 1990), pneumonia (Crandell *et al.*, 1977; Humphrey, 1982; Andrews *et al.*, 1985; Little, 1986; Corbeil *et al.*, 1986; Gogolewski *et al.*, 1987a; Bryson, *et al.*, 1990), pleuritis (Humphrey, 1982; Blood *et al.*, 1983; Little, 1986; Harland *et al.*, 1990), abortions (Humphrey, 1982; Corbeil *et al.*, 1986; Corbeil *et al.*, 1987; Harris and Janzen, 1989), and synovitis and conjunctivitis (Humphrey, 1982; Blood *et al.*, 1983; Harris and Janzen, 1989; Barnum, 1990). Due to the association of *H. somnus* with a variety of disease conditions in cattle, this disease syndrome is collectively known as *H. somnus* complex. Further, *H. somnus* has been shown to inhabit the reproductive and upper respiratory tract of healthy cattle (Corstvet *et al.*, 1973; Humphrey *et al.*, 1982b; Little, 1986).

Pathogenic bacteria have developed a variety of mechanisms for evading destruction by host defence mechanisms (Densen and Mandell, 1980). The ability to survive and multiply within cells of the mononuclear phagocyte system is a strategy that has been used by a number of important pathogens of humans and domestic animal, including Mycobacteria (Lurie, 1942), *Brucella* (Smith and Fitzgeorge, 1964),

Salmonella (Furness, 1958), *Yersinia* (Cavanaugh and Randall, 1959), and *Listeria* (Mackaness, 1964) species. By surviving inside phagocytes, these microbes are protected from the immune system and able to cause local and systemic infections. It has been reported that *H. somnus* was able to survive and multiply in bovine alveolar macrophages and blood monocytes (Lederer *et al.*, 1987), but the interaction between *H. somnus* and bovine mononuclear cells was poorly understood.

The survival or multiplication of *H. somnus* in mononuclear phagocytes *in vitro* provides a model in which the microbicidal or inhibitory capacity of infected cells or cytokine treated cells may be studied further. The objective of this study was to further characterize the nature of interaction between *H. somnus* and bovine mononuclear phagocytes.

MATERIALS AND METHODS

Bacteria

A pathogenic strain of *H. somnus* (HS25) originally isolated from the pneumonic lung of a calf was obtained from Alberta Agriculture in Edmonton, Alberta was maintained in egg yolk, at -70C. Egg yolk stock culture was made by inoculating the pure cultures into 6-7 day old embryonated chicken eggs and incubating them at 37C until death of embryo occurred 1-3 days later. The egg yolks were then removed under sterile conditions, aliquoted, and stored at -70C. The isolate HS25 was selected for this study based on past research at V.I.D.O., including studies of interaction between PMN and *H. somnus* (Pfeifer, 1992), and bovine animal model studies (Harland *et al.*, 1990). Before each experiment, an aliquot of egg yolk stock culture

was thawed and spread onto a 5% sheep blood agar plate (PML Microbiologicals, Richmond, B.C.) and incubated for 36 h at 37C in an atmosphere containing 5% CO_2. A loopful of colonies was transferred to BHITT [Brain Heart Infusion (Difco, Detroit, MI) supplemented with 1 µg/ml of 1% thiamine monophosphate (Sigma, St. Louis, MO), and 10 µg/ml of 10% Trizma base (Sigma, St. Louis, MO)], and incubated aerobically for 10 h at 37C on a shaker. As the final step, this broth culture was diluted to 1:4 with BHITT and incubated for 2 h until the optical density (OD) (660 nm) reached 0.4. One ml of this broth was found to contain approximately $1X10^9$ logarithmically growing *H. somnus*. Stationary phase *H. somnus* cultures were aerobically grown for 24 h at 37C in 10 ml of BHITT on a shaker. One ml of this broth contained approximately $1X10^9$ stationary phase *H. somnus*. The actual number of bacteria per ml was determined by plating 10-fold serial dilutions of the suspension in duplicate and counting colonies. This number was used to calculate the ratio of phagocytes to bacteria in all experiments. *H. somnus* was recovered and washed twice with cold (4C) Hanks balanced salt solution (HBSS) by centrifugation before being used.

The culture of *Staphylococcus aureus* used was originally isolated from a case of bovine mastitis and was subsequently frozen at -70C in skim milk as a pure stock culture. A sample of stock culture was thawed and spread on a 5% sheep blood agar plate and incubated for 24 h at 37C in an atmosphere containing 5% CO_2. After incubation, a colony of *S. aureus* was selected, and incubated in 100 ml of Luria-Bertani (LB) broth (Difco, Detroit, MI) that was grown aerobically for 18 h at 37C on a shaker. One ml of this seed culture was mixed with 100 ml of LB-Broth and

incubated for 6 h at 37C on a shaker. Approximately $1X10^9$ CFU of *S. aureus* were in 1 ml of this broth.

Source and Isolation of Bovine Mononuclear Phagocytes

Bovine alveolar macrophages (BAM) and bovine blood monocytes (BBM) were obtained from clinically normal, 2-3 month old dairy and 6-12 month old beef calves. These animals were maintained at V.I.D.O. research facilities at the University of Saskatchewan. Heparinized (Organon Teknika, Toronto, Canada) blood was centrifuged for 5 min at 37C and 1000 g to separate the buffy coat from the platelet rich plasma. The buffy coat was removed and diluted with HBSS and layered on Ficoll-Paque (Pharmacia LKB, Biotechnology, Uppsala, Sweden) gradient to obtain mononuclear cell-rich population. The cells from the interface were removed and washed twice with HBSS. Isolated peripheral blood mononuclear cells (PBMC) were suspended in macrophage SFM-medium (serum free medium) with glutamine and without antibiotics (Gibco BRL, Life Technologies Inc. Grand Island, NY) supplemented with 10% fetal bovine serum (heat inactivated) (Gibco BRL, Life Technologies Inc. Grand Island, NY). Mononuclear cell viability was determined by trypan blue exclusion, and averaged 95% or better. PBMC suspensions contained between 4-10% monocytes as determined by microscopic examination of stained smears (Diff-Quick Fixative, Baxter Healthcare Corporation, IL).

Isolation of Bovine Alveolar Macrophages

Lung lavages were performed using a endotracheal tube on anesthetized calves.

Three hundred ml of warm (37C) HBSS solution were used for each lavage. Lavage fluid was filtered with a nylon strainer after recovery and centrifuged at 4C for 20 min and 800 g. Cell pellets were suspended in HBSS and layered on Ficoll-Paque gradient to obtain mononuclear cells. Mononuclear cells were removed in the interphase and washed twice with HBSS and suspended in macrophage medium. Alveolar macrophage viability, determined by trypan blue exclusion, averaged 95%. Lung cell suspensions contained between 65%-85% macrophages as determined by microscopic examination of stained smears (Diff-Quick Fixative, Baxter Healthcare Corporation, IL).

The *H. somnus* Bovine Experimental Model

A field isolate of *H. somnus* (HS25) was injected intravenously into 6-8 month old beef calves (Harland *et al.*, 1990; Schuh *et al.*, 1991). A challenge dose contained approximately $5X10^7$ colony forming units (CFU). Clinical signs occurring after challenge included fever, depression, and polyarthritis. The challenge was fatal to 60%-70% of the calves and the necropsy findings included meningitis, encephalitis (Gomis *et al.*, 1993), myocarditis, pericarditis, pneumonia, pleuritis and polyarthritis. This bovine *H. somnus* model closely reproduced the clinical and pathological features of the disease as seen in Western Canada.

Blood samples were taken from these experimentally infected animals daily for the colorimetric bactericidal assay and ^3H uracil assay.

Recombinant Bovine Cytokines

Recombinant bovine IL-1β (rBoIL-1β) (American Cyanamid Co., Princeton, NJ)

was produced in an *E. coli* expression system. The stock solution of 6.8 mg of rBoIL-1β/ml had a specific activity of $7.7X10^6$ IU/mg protein as determined by a murine thymocyte proliferation assay. There was 125 EU of endotoxin/mg of protein as determined by the limulus amoebocyte lysate assay (Whittacker Corp., Walkersville, MD). The rBo granulocyte-macrophage colony stimulating factor (rBoGM-CSF) (American Cyanamid Co., Princeton, NJ) was produced in an *E. coli* expression system. Recombinant BoGM-CSF had a concentration of 2.7 mg/ml with an endotoxin content of 30.2 EU/mg of protein, and specific activity of $11.8X10^6$ U/mg (50% proliferation of neonatal bovine bone marrow). Recombinant bovine IFN-γ (rBoIFN-γ) (Ciba-Geigy, Basel, Switzerland) had a protein concentration of 0.54 mg/ml, > 95% purity, and the specific activity was $3.0X10^{(6)}$ U/mg protein. The endotoxin content was 1.0 ng/mg protein. Recombinant bovine TNF-α (rBoTNF-α) (Genentech Inc., San Francisco, CA) was produced in an *E. coli* expression system. Recombinant BoTNF-α had a protein concentration of 0.4 mg/ml, and specific activity was $1X10^{(6)}$ U/mg protein as determined by cytotoxicity of L929 cells. For the colorimetric bactericidal assay, 100 ng/ml of individual cytokine and 5 μg/ml of *E. coli* LPS (serotype 0111:B4) (Sigma Chemical Co. St. Louis, MO) were added to the macrophage medium to activate bovine mononuclear phagocytes.

Colorimetric Bactericidal Assay

The colorimetric bactericidal assay using MTT was a modification of a technique used by Stevens *et al.*, (1991). This assay was conducted in round bottom, 96 well microtitre plates (Nunclon, Roskilde, Denmark). To each microtitre well,

$1X10^6$ PBMC or BAM (in 50 µl of macrophage medium), and *H. somnus* (in 20 µl of HBSS) were added and incubated for 2 h at 37C in an atmosphere containing 5% CO_2 to allow macrophages to phagocytize *H. somnus* and adhere to the bottom of the microtitre wells. Three different ratios of phagocyte to bacteria were established (1:1, 1:10, and 1:100). Previous analyses established that BBM or BAM can phagocytize unopsonized *H. somnus*. After 2 h incubation, microtitre wells were washed twice with HBSS to remove non-adherent cells and extracellular bacteria, and 100 µl of macrophage medium containing 1 µg/ml Gentamycin (Gibco BRL, Life Technologies Inc. Grand Island, NY) was added. Isolated PBMC or BAM without *H. somnus* were used as controls. All samples were prepared in quadruplicate. The plates were incubated at 37C in an atmosphere containing 5% CO_2 for 0, 6, 18, 24, 30, 42, 48 h before reading the samples by the colorimetric assay. At the end of each time point, 50 µl of 2% saponin was added to the plates to lyse eukaryotic cells and 50 µl of 2 mg/ml of MTT (Sigma Chemical Co. St. Louis, MO) was added and incubated for 30 min at 37C in an atmosphere containing 5% CO_2 to allow viable *H. somnus* to reduce MTT to purple formazan. Then-MTT containing plates were centrifuged for 10 min at 2000 g to pellet formazan-laden *H. somnus*. The supernatant was removed by suction using a 25_G 5/8 inch hypodermic needle. As the final step, all wells received 150 µl of isopropanol (BDH Inc. Toronto, ON) were mixed vigorously by pipetting and plates were allowed to sit for 10 min at room temperature. Formazan produced by bacteria was quantified by measuring OD with wavelength of 595 nm (reference 655 nm). The OD readings were obtained using an automated 96-channel microtitre plate reader (Bio-Rad Laboratories, model 3550, Richmond, CA). At time 0 h and 48 h, samples from

microtitre plates were cultured on 5% sheep blood agar plates in duplicate to determine the identity of bacterial colonies. A standard curve of bacterial viability was prepared from *H. somnus* suspensions. *S. aureus* was used to determine the bactericidal ability of BBM or BAM in this colorimetric assay. Bacteriological studies were conducted at Western College of Veterinary Medicine, University of Saskatchewan. A score from 0 to 4+ was assigned on the presence of *H. somnus* colonies on 5% sheep blood agar plates in none, 25%, 50%, 75%, and 100% of the area on blood agar plate. In order to study the bactericidal ability of BBM, opsonized *S. aureus* was used in this assay. Anti-*S. aureus* bovine convalescent sera came from animals that had recovered from *S. aureus* mastitis.

^{3}H Uracil Assay

The assay procedure used to study this interaction was a modification of the assay used by Pfefferkorn and Pfefferkorn (1981). This assay was conducted in round bottom, 96 well microtitre plates (Nunclon, Roskilde, Denmark). Each well contained $3X10^{6}$ PBMC or $1X10^{6}$ BAM in 100 µl of macrophage SFM-medium (serum free medium) with glutamine (Gibco BRL, Life Technologies Inc. Grand Island, NY). The microtitre plates were incubated for 2 h at 37C in an atmosphere containing 5% CO_2 to allow monocytes or alveolar macrophages to adhere to the bottom of the wells. Then the microtitre plates were washed 4 times with HBSS to remove non-adherent cells. As the next step, *H. somnus* was added to the microtitre wells and centrifuged for 5 min at 300 g and incubated for 3 h at 37C in an atmosphere containing 5% CO_2 to allow *H. somnus* to infect BBM or BAM. Three different ratios of BBM or BAM to

H. somnus were established (1:1, 1:0.1 and 1:0.01). Three control samples contained *H. somnus* alone according to the above ratios. One control sample contained macrophages alone and another control was a medium control. All samples were prepared in triplicate. After incubating macrophages with *H. somnus* for 3 h, plates were washed 4 times with macrophage medium to remove extracellular bacteria and then all wells pulsed with 1 μCi (in 200 μl of macrophage medium) of ^{3}H uracil (Amersham, Canada Ltd. Oakville, ON) and incubated for 12 h at 37C in an atmosphere containing 5% CO_2. To measure ^{3}H uracil incorporation into nucleic acids, macrophages were harvested (using distilled water containing 0.02% sodium azide [Sigma Chemicals Co. St. Louis, MO]) onto filtermats (SkatronAS Sterling, VA) by using a 12 well semiautomatic cell harvester (SkatronAS, Norway). These filtermats were then air dried and inserted into 5 ml scintillation vials (Skatron, Norway) and 1 ml of liquid scintillation cocktail (Beckman Instrument Inc. Fullerton, CA) was added. Radioactivity was measured by a beta counter (Beckman LS 1701, Liquid Scintillation System, Fullerton, CA).

Ultrastructural Studies

PBMC or BAM were incubated with *H. somnus* at 37C in an atmosphere containing 5% CO_2 for 2 h at a ratio of 1:10 (phagocytes : bacteria), in 0.5 ml of macrophage medium in 14 ml 17X100 mm polypropylene tubes (Becton Dickinson, NJ) on a nutator (Clay Adams, Becton Dickinson Co, NJ). After centrifugation, the cell pellets were washed four times with HBSS to remove extracellular bacteria. As the next step, 10 ml of macrophage medium supplemented with 1 μg/ml Gentamycin

(Gibco BRL, Life Technologies Inc. Grand Island, NY) was added to the tubes, which were then incubated at 37C in an atmosphere containing 5% CO_2 for 0, 6, 18 and 24 h on a nutator. At the end of each time point, tubes were centrifuged for 10 min at 300 g; the supernatant was discarded and the cell pellet was resuspended in glutaraldehyde (Sigma, St. Louis, MO). Transmission electron microscopic studies of the cell pellets were conducted at Western College of Veterinary Medicine, University of Saskatchewan. Samples from polypropylene tubes at each time point were cultured on 5% sheep blood agar plates in duplicate to determine the purity of *H. somnus* colonies. Alveolar macrophages were incubated with macrophage medium supplemented with 10% antibiotic-antimycotic solution (Sigma Chemical Co. St. Louis, MO) (10,000 units of penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml in 0.9% sodium chloride) for 2 h at 37C in an atmosphere containing 5% CO_2 to eliminate contaminants before beginning of the infection studies with *H. somnus* for ultrastructural studies.

Statistical Analysis

Analysis of variance (ANOVA) was executed using Minitab (Minitab for Windows, ver, 9.2, Minitab Inc., 3081, Enterprise Drive, State College, PA) in a Randomized Complete Block Design, to test the significance of the treatments. Statistical significance was assumed at $P \leq 0.05$. Mean comparisons were carried out using Lsd (least significant difference) test whenever the F test for treatment was significant at the 5% level.

RESULTS

Standard Curve for Colorimetric Bacterial Assay

Conversion of MTT to formazan by *H. somnus* was highly correlated (r=0.993) with the number of viable bacteria (Fig. 1). Addition of 50 µl of 2% saponin to the colorimetric assay had no effect on viability of *H. somnus* (data not shown). Control samples containing BBM or BAM alone had no production of formazan after cells were lysed with 2% saponin. The bactericidal activity of BBM or BAM was measured by using opsonized *S. aureus* in this assay (Fig. 2). BBM or BAM were able to kill 100% of ingested *S. aureus* within 24 h (data not shown see Fig. 2). Optical density values below 0.02 had no correlation with CFU of *H. somnus* (data not shown), a limitation of this assay procedure.

Survival of *H. somnus* in BBM and BAM

Survival of *H. somnus* in BBM (Fig. 3) and BAM (Fig. 4) was demonstrated in the colorimetric bactericidal assay. Figure 5 shows the survival *H. somnus* both in *in vitro* infected BBM and BBM obtained from experimentally infected cattle. The colorimetric assay procedure was identical in both *in vitro* and *ex vivo* assay, except that there was no *in vitro* addition of *H. somnus* to the latter procedure. Also, no gentamycin was added to the *ex vivo* assay, since no extracellular *H. somnus* were demonstrated by either microscopic or ultrastructural examination of cell pellets. The concentration of gentamycin in the assay medium (1 µg/ml), based on previous studies indicating that gentamycin does not readily cross the bovine monocyte cell membrane (Dees *et al.*, 1985; Lederer, *et al.*, 1987), was able to inhibit extracellular growth of

H. somnus in this colorimetric assay (data not shown). The number of CFU of *H. somnus* was between 5-20 in *ex vivo* experiments at time 0 h, compared to over $1X10^6$ CFU at 24 h in the *in vitro* colorimetric assay. This observation confirmed the ability of *H. somnus* to grow in BBM as compared to *H. somnus* survival in *in vitro* assay system. Gentamycin was not added to the macrophage medium to control extracellular bacteria in this *ex vivo* assay, the kinetics of extracellular bacterial growth were distinct from intracellular growth (data not shown).

Effect of Cytokine Treatment on BBM

The kinetics of *H. somnus* survival in cytokine treated BBM are demonstrated in Fig. 6. Treatment of BBM with rBoIL-1β (0.085 ± 0.037), rBoIFN-γ (0.10 ± .037), rBoGM-CSF (0.062 ± 0.065), rBoTNF-α (0.101 ± 0.015), and *E. coli* LPS (0.060 ± 0.017) caused a significant difference in OD values at 18 h ($P<0.006$) (control=0.149 ± 0.028) where the highest intracellular bacterial growth occurred (Fig. 6). No significant difference of OD values occurred at 24 h ($P<0.085$) but, again, a significant difference occurred at 48 h for rBoINF-γ (0.048 ± 0.009), rBoGM-CSF (0.042 ± 0.014), and *E. coli LPS* (0.059 ± 0.021) ($P<0.005$) (control=0.089 ± 0.039) treated BBM (Fig. 6). However, none of these treatments were able to completely eliminate the intracellular *H. somnus* from BBM. *H. somnus* was able to survive in cytokine and *E.coli* LPS treated BBM at all time points as measured by CFU of *H. somnus* on blood agar plates. Recombinant bovine cytokines and *E. coli* LPS had no direct effect on viability of either *H. somnus* or BBM alone during the entire length of colorimetric assay procedure (data not shown). Moreover, *H. somnus* had no effect on viability of

BBM due to it's survival in BBM over the assay period (data not shown).

Ultrastructural Demonstration of *H. somnus* in BBM and BAM

Survival of *H. somnus* in both BBM and BAM was demonstrated at 2, 6, 18 and 24 h (Fig. 7, 8 and 9) after infection with *H. somnus*. At 2 h, most of the bacteria were surrounded by pseudopodia of phagocytes. Bacteria were tightly bound by membranes when they were inside both BBM and BAM. The intracellular bacterial load was increased at 7 and 24 h of infection (Fig. 8 and 9). *H. somnus* was able to survive and multiply in both BBM and BAM even after 24 h of infection as measured by CFU of *H. somnus* on blood agar plates (data not shown).

^{3}H Uracil Incorporation into Nucleic Acids (^{3}H Uracil Assay)

This assay detects the ^{3}H uracil uptake by *H. somnus* since uracil is an essential requirement for growth of *H. somnus*. ^{3}H uracil incorporation into nucleic acids of *H. somnus* containing phagocytes was detectable in this assay system (Fig. 10, and 11). In general, BAM containing *H. somnus* had higher counts per minutes (CPM) at a ratio of 1:1 alveolar macrophages to *H. somnus* (15761 ± 5326) ($P<0.015$) than BBM containing *H. somnus* at the ratio of 1:0.1 (1304 - 1295) ($P<0.021$). Figure 12A shows ^{3}H uracil uptake by BBM, that were recovered from cattle experimentally infected with *H. somnus*. In this *ex vivo* assay system, animals at day 9 post-infection had significantly higher CPM (9810 ± 7241) ($P< 0.009$) (back ground CPM was < 200) than at other times. Furthermore, in this *ex vivo* assay system, *H. somnus* originated from BBM as opposed to *in vitro* addition of *H. somnus*. The kinetics of *H. somnus*

isolation from buffy coat were demonstrated in Fig. 12B. The bacteremia of experimentally infected cattle with *H. somnus* follows a multiphasic pattern with unpredictable intervals between phases (Harland, R. personal communication). This multiphasic pattern with unpredictable intervals between phases corresponds with the ^{3}H uracil uptake by BBM (compare day 9 in Fig. 12A and 12B).

DISCUSSION

Since uracil is an essential nutrient for *H. somnus*, (Inzana and Corbeil, 1987) ^{3}H uracil uptake by *H. somnus* was used as a tool to study the intracellular activity of this bacterium after ingestion by BBM. The results of this assay revealed that measurement of ^{3}H uracil uptake provides a means whereby growth inhibition or survival of *H. somnus* could be studied in BBM. The ^{3}H uracil assay demonstrated the incorporation of ^{3}H uracil into BBM or BAM that had ingested *H. somnus*. In this assay system, uracil could either be incorporated into the nucleic acids of BBM or into *H. somnus*. Since there were no eucaryotic cellular function inhibition studies, it is not possible to rule out these two possibilities.

The location, survival and growth of *H. somnus* in BBM was studied by using the MTT colorimetric assay and ultrastructural studies. This assay is based on the principle that live bacteria convert MTT intracellularly to purple formazan in direct proportion to the number of viable cells. Bacterial survival, growth or killing by mononuclear phagocytes is then measured by colorimetric determination of formazan production by live bacteria. This colorimetric bactericidal assay is more rapid, more sensitive, and less expensive than traditional bactericidal assays that use colony

counting of bacteria on nutrient agar plates (Stevens *et al.*, 1991). Using this assay system, it was found that *H. somnus* was able to survive both in BBM and BAM. Moreover, it was possible to demonstrate the growth of *H. somnus* in BBM isolated from cattle experimentally infected with *H. somnus*. The intracellular growth of *H. somnus* in BBM was confirmed by using CFU of *H. somnus* rather than using OD values of MTT assay. One limitation of this assay was the inability to extrapolate CFU of *H. somnus* when OD values were below 0.02.

Following infection, the number of intracellular bacteria initially decreased (Fig. 6). In the first 6 h, about 40% of *H. somnus* were killed, presumably because of killing mechanisms of BBM. Activation of BBM might occur nonspecifically as a result of the presence of traces of LPS, adherence of BBM to the plastic, or as a result of presence of the *H. somnus*. [*H. somnus* is a Gram-negative organism whose cell wall contains LOS (lipooligosaccharide)]. The decline in the number of bacteria may also, in part, represent an inability of some *H. somnus* to adapt to intracellular growth. Following this initial decrease, however, the surviving *H. somnus* begin to replicate in BBM and the number of intracellular *H. somnus* increased until 24-48 h post-infection (Fig. 6). Optical density values did not increase beyond 0.3 at the peak growth, probably as a result of overburden of macrophages with *H. somnus*, which may subsequently cause the death of the BBM. These *in vitro* and *ex vivo* observations confirmed the ability of *H. somnus* to survive and multiply in macrophages.

Macrophage activation and induction of antimicrobial mechanisms by cytokines is complex, partially as a result of multiple pathways used for microbial killing. IFN-γ is capable of up regulating three major pathways described for microbial killing, i.e.,

the production of reactive oxygen intermediates (Nathan *et al.*, 1983), the production of nitrogen intermediates (Stuehr and Marietta, 1987), and the induction of indolamine 2,3-dioxygenase to catabolize tryptophan (Carlin *et al.*, 1989). IFN-γ has been shown to induce antimicrobial activities against a variety of intracellular parasites, including *Mycobacterium bovis* (Flesch and Kaufmann, 1988), *Mycobacterium tuberculosis* (Rook *et al.*, 1986; Flesch and Kaufmann, 1988), *Leishmania major* (Douvas *et al.*, 1985; Passwell *et al.*, 1986; Green *et al.*, 1990), and *Listeria monocytogenes* (Portoy *et al.*, 1989). The ability of other cytokines to activate macrophage antimicrobial activities is more variable. TNF-α-treated macrophages inhibit the survival or growth of *Mycobacterium lepraemurium* (Denis, 1991), and *Mycobacterium avium* (Bermudez and Young, 1988; Denis, 1991). GM-CSF increases the production of H_2O_2 (Ding *et al.*, 1988) by macrophages and activates these cells to inhibit the growth of *Trypanosoma cruzi* (Reed *et al.*, 1987) and to kill *Leishmania donovani* (Weiser *et al.*, 1987). IL-1 plays a role in macrophage activation (Philip and Epstein 1986; Chen *et al.*, 1987) and, when administered to mice, prevents chronic infection by *Brucella abortus* (Zhan *et al.*, 1991). Treatment of BBM with recombinant bovine cytokines and *E.coli* LPS caused a significant effect on BBM to reduce their bacterial load at 18 h, where the highest bacterial growth occurred in the control (Fig. 6). No significant difference of OD values occurred at 24 h, but again significant differences occurred at 48 h for rBoINF-γ, rBoGM-CSF, and *E. coli LPS* treated BBM (Fig. 6). None of these treatments were able to completely eliminate intracellular *H. somnus* in BBM. Pre-treatment of BBM with cytokines was not performed in this study. Furthermore, it is not known whether *H. somnus* has an ability to deactivate macrophages, even

though the BBM had been treated with recombinant bovine cytokines *and E. coli* LPS.

Ultrastructural studies conducted on BBM or BAM pellets infected with *H. somnus* demonstrated intracellular *H. somnus*. The bacteria tended to occur in discrete vacuoles, with membranes tightly opposed to the bacterial surface. Further, viability of *H. somnus* was not affected by the interaction with either BBM or BAM for 24 h. BBM or BAM cell pellets were not incubated for more than 24 h because of overcrowding effects of eucaryotic cells in polypropylene tubes. Microtitre plates containing BBM or BAM monolayers infected with *H. somnus* were not used for ultrastructural studies due to the inability to recover eucaryotic cells from the plastic surface.

Fig. 1: The relationship between colony forming units (CFU) values of live *H. somnus* and optical density (OD) reading of MTT colorimetric assay. Live *H. somnus* was serially diluted in macrophage medium to obtain OD values. The correlation coefficient (r) of CFU values of live *H. somnus* and OD values of MTT colorimetric assay was 0.993.

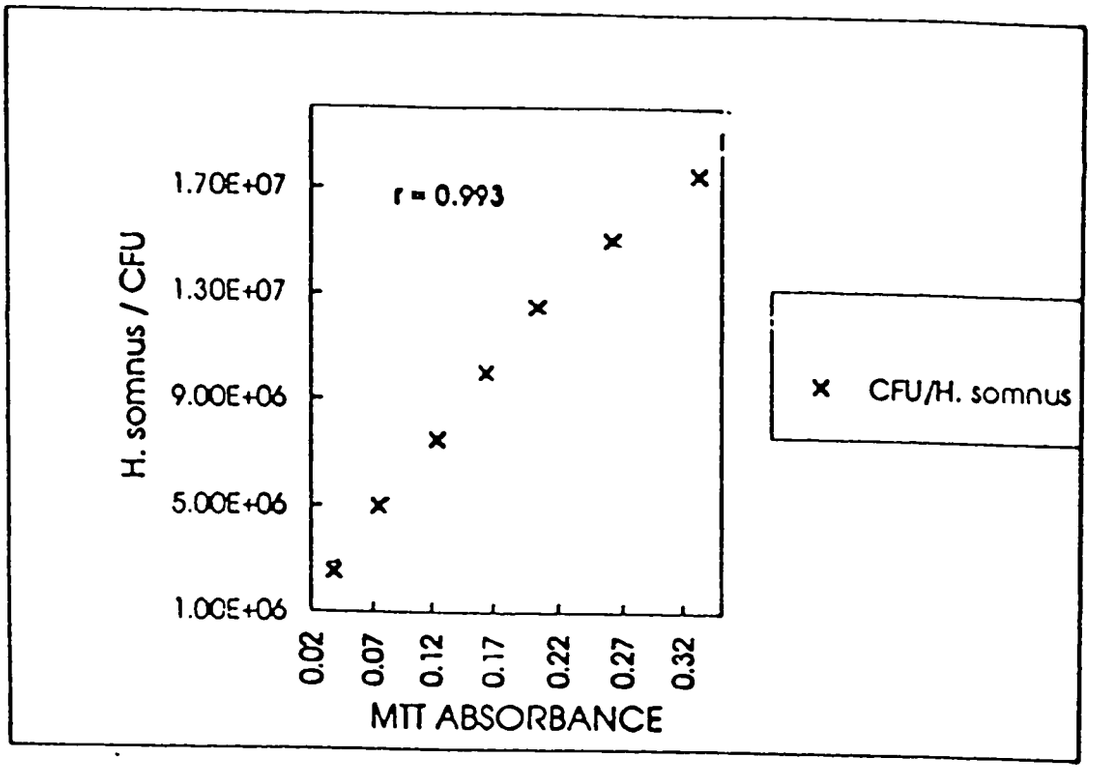

1.70E+07
1.30E+07
9.00E+06
5.00E+06
1.00E+06
r = 0.993
H. somnus / CFU
0.02
0.07
0.12
0.17
0.22
0.27
0.32
MTT ABSORBANCE
CFU/H. somnus

Fig. 2: Bactericidal capacity of bovine blood monocytes BBM with the interaction of *H. somnus* as measured by colorimetric bactericidal assay. Note 100% destruction of *S. aureus* by BBM in control samples within 24 h of infection, *H. somnus* numbers were initially depressed, but the bacteria survived and then multiplied. Each time point value shown is the mean and standard deviation. (n=5)

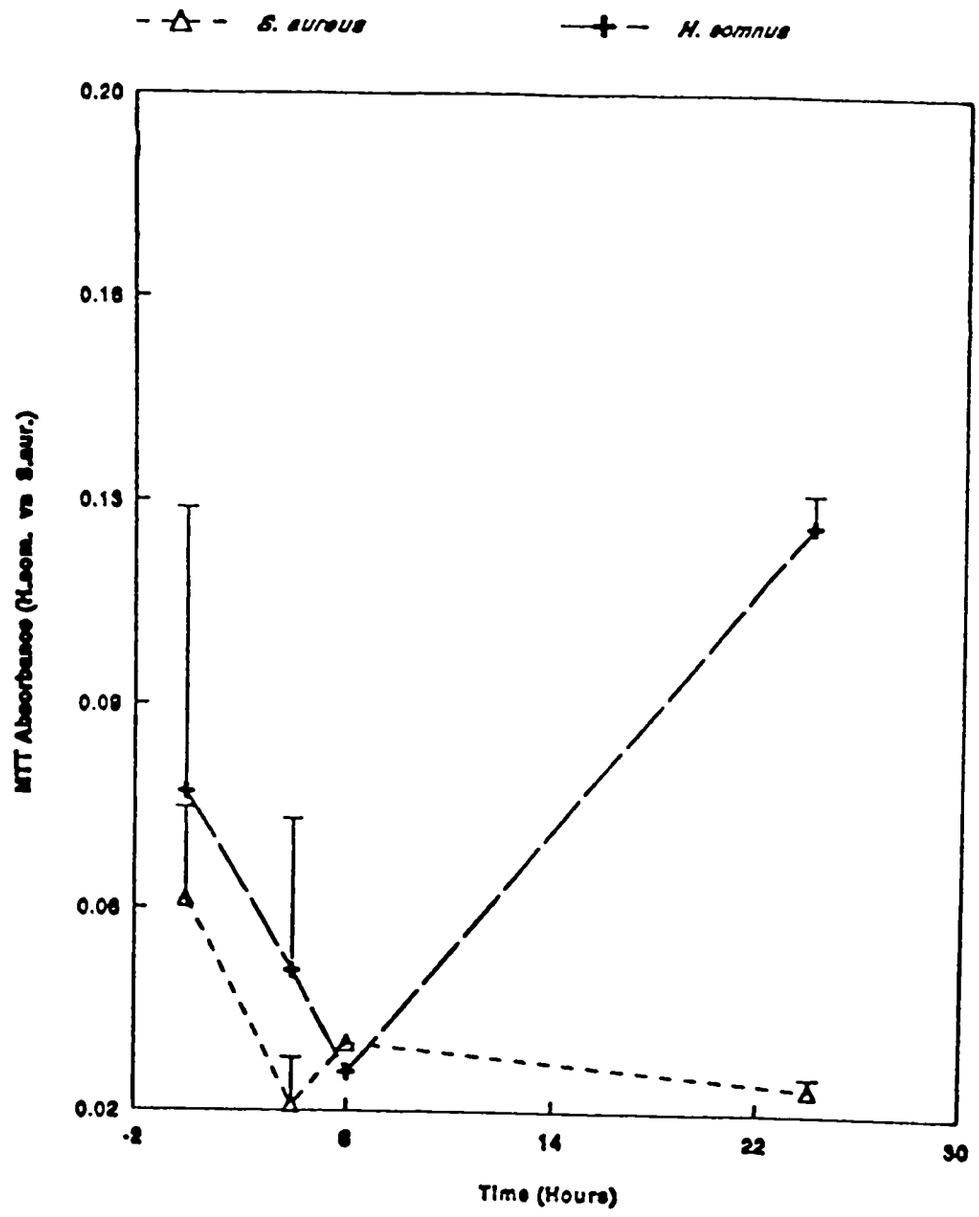

S. aureus
H. somnus
0.20
0.16
0.12
0.08
0.04
0.02
MTT Absorbance (H.som. vs S.aur.)
-2
6
14
22
30
Time (Hours)

Fig.3: Intracellular survival of *H. somnus* in bovine blood monocytes (BBM) as demonstrated by colorimetric bactericidal assay. Phagocytes to bacteria ratio was 1:10 at the beginning of the assay. Each time point value shown is the mean and standard deviation. The right Y axis indicated the CFU of *H. somnus* corresponding to the OD values of left Y axis. (n=5)

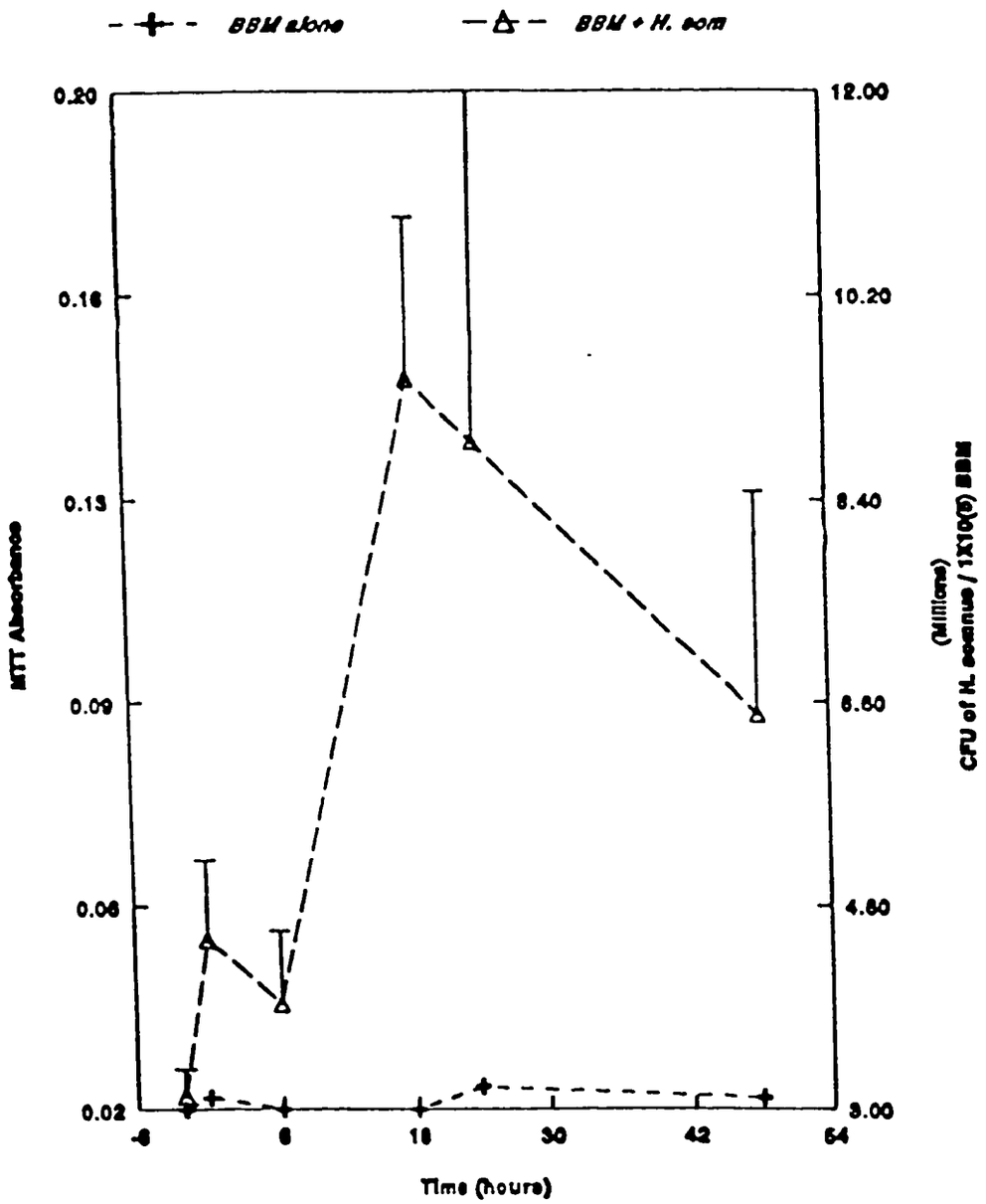

BBM alone
BBM + H. ecm
0.20
0.16
0.13
0.09
0.06
0.02
12.00
10.20
8.40
6.60
4.80
3.00
MTT Absorbance
CFU of H. ecanus / 1X10(5) BBM
(Millions)
-6
6
18
30
42
54
Time (hours)

Fig. 4: Intracellular survival of *H. somnus* in bovine alveolar macrophages (BAM) as demonstrated by colorimetric bactericidal assay. Phagocytes to bacteria ratio was 1:10 at the beginning of the assay. Each time point value shown is the mean and standard deviation. The right Y axis indicated the CFU of *H. somnus* corresponding to the OD values of left Y axis. (n=5)

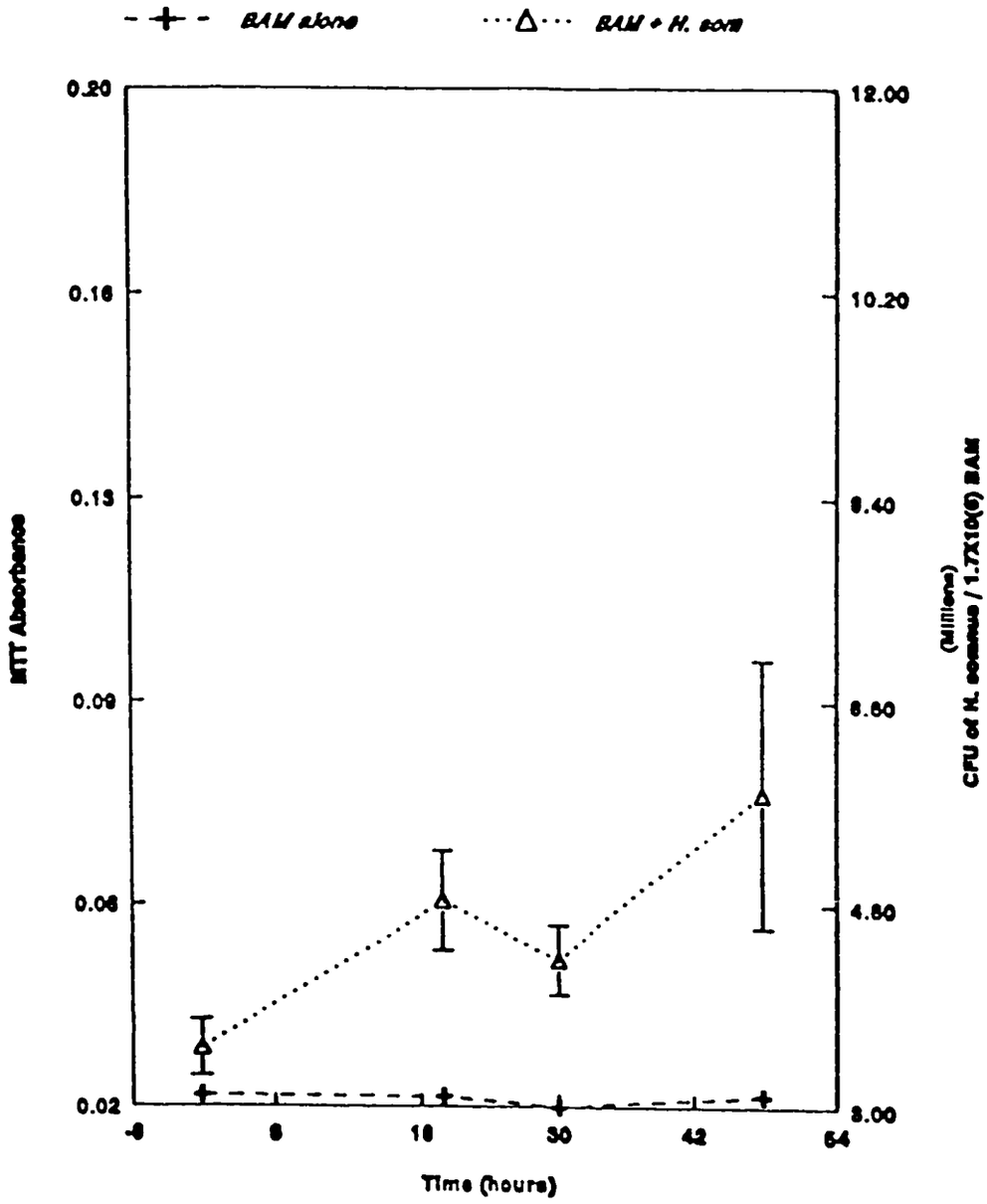

BAM alone
BAM + H. somni
0.20
0.16
0.13
0.09
0.06
0.02
MTT Absorbance
12.00
10.20
8.40
6.60
4.80
3.00
CFU of H. somnus / 1.7X10(6) BAM
(Millions)
-6
6
18
30
42
54
Time (hours)

Fig. 5: MTT assay demonstration of intracellular survival of *H. somnus* in *in vitro* (n=5) and *ex vivo* (n=4) experiments. In *in vitro* assay live *H. somnus* were added to bovine blood monocytes (BBM), while *ex vivo* assay was conducted using isolated BBM from cattle experimentally infected with *H. somnus* without further addition of *H. somnus*. (In *ex vivo* assay *H. somnus* originated from isolated BBM.) Phagocytes to bacteria ratio was 1:10 at the beginning of the *in vitro* assay. Each time point value shown is the mean and standard deviation. The right Y axis indicated the CFU of *H. somnus* corresponding to the OD values of left Y axis.

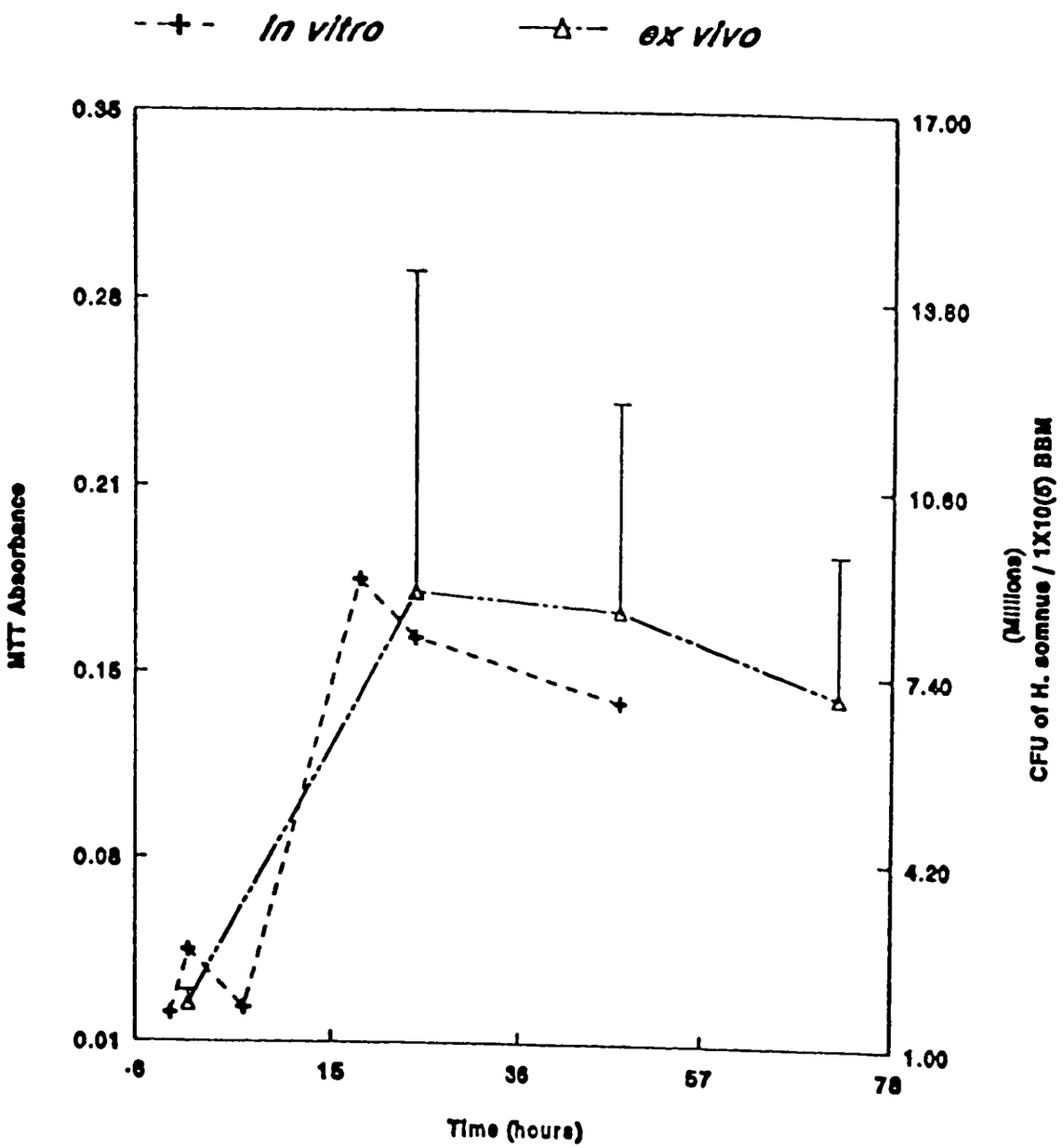

In vitro
ex vivo
0.35
0.28
0.21
0.15
0.08
0.01
MTT Absorbance
17.00
13.80
10.60
7.40
4.20
1.00
CFU of H. somnus / 1X10(5) BBM
(Millions)
-6
15
36
57
78
Time (hours)

Fig. 6: Intracellular survival kinetics of *H. somnus* in bovine blood monocytes (BBM) treated with different recombinant bovine cytokines and *E. coli* LPS. Live *H. somnus* and 100 ng/ml recombinant cytokines (IFN-γ, TNF-α, IL-1β or GM-CSF) or *E. coli* LPS (5μg/ml) added to BBM at the beginning of the colorimetric assay. The right Y axis indicated the CFU of *H. somnus* corresponding to the OD values of left Y axis. (n=5) Each time point value shown is the mean and standard deviation.

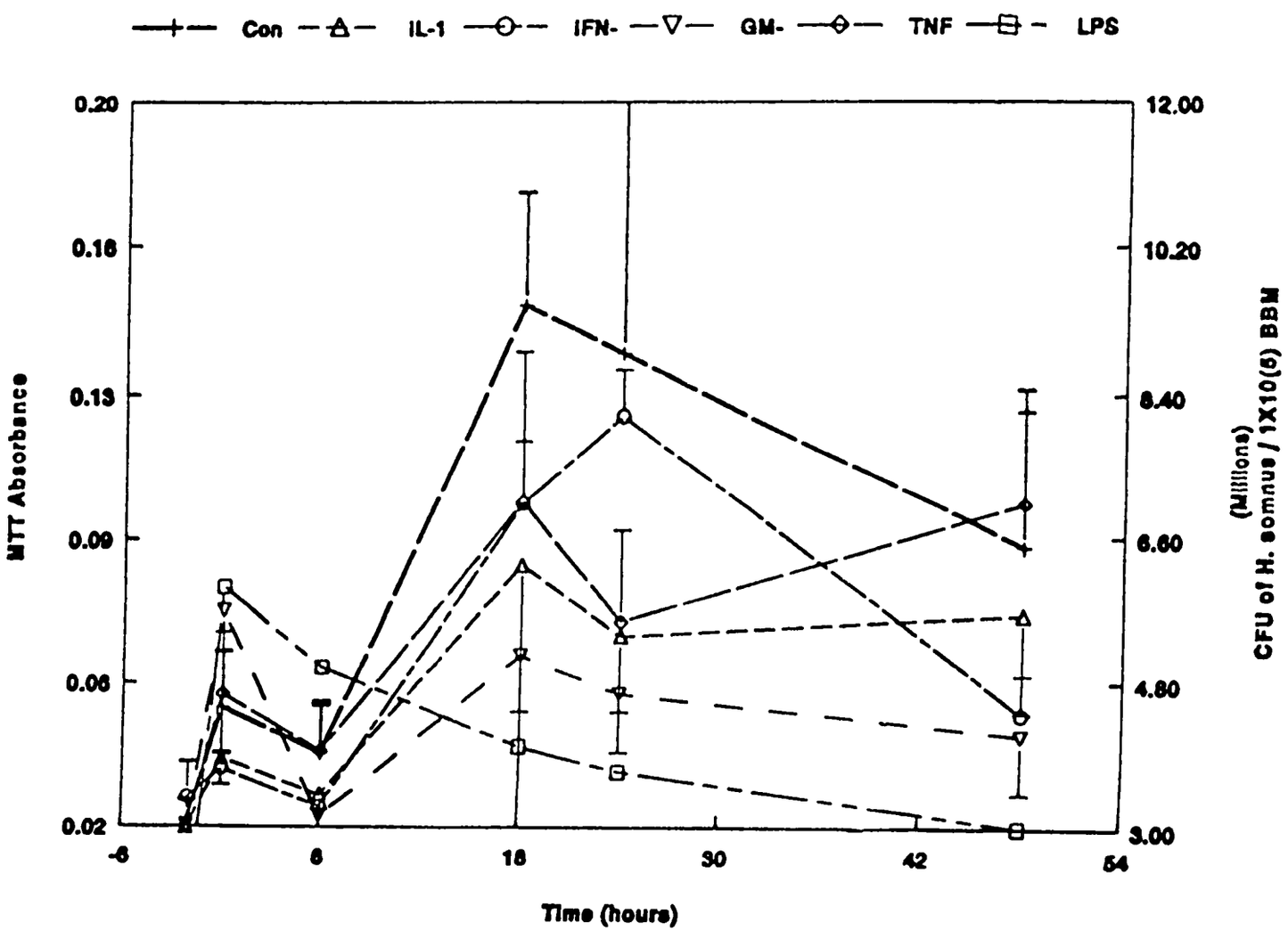

Con
IL-1
IFN-
GM-
TNF
LPS
0.20
0.16
0.13
0.09
0.06
0.02
MTT Absorbance
12.00
10.20
8.40
6.60
4.80
3.00
CFU of H. somnus / 1X10(6) BBM
(Millions)
-6
6
18
30
42
54
Time (hours)

Fig. 7: Ultrastructural demonstration of *H. somnus* in bovine blood monocytes (BBM). *H. somnus* added to BBM in polypropylene tubes and incubated for 2 h; then cell pellets were washed two times to remove extracellular bacteria and added the macrophage medium containing gentamycin to control the extracellular bacteria. (A, and B micrographs indicating 2 h after infection with *H. somnus*) (A) Some of the bacteria are surrounded by pseudopodia. (B) Some of the bacteria have been internalized by BBM. Note that bacteria are in individual vacuoles with tightly bound membranes. (C, and D micrographs indicating 7 h after infection with *H. somnus*) [C: and D:] Multiple bacteria are present in vacuoles of BBM. [Bar = 0.05μm]

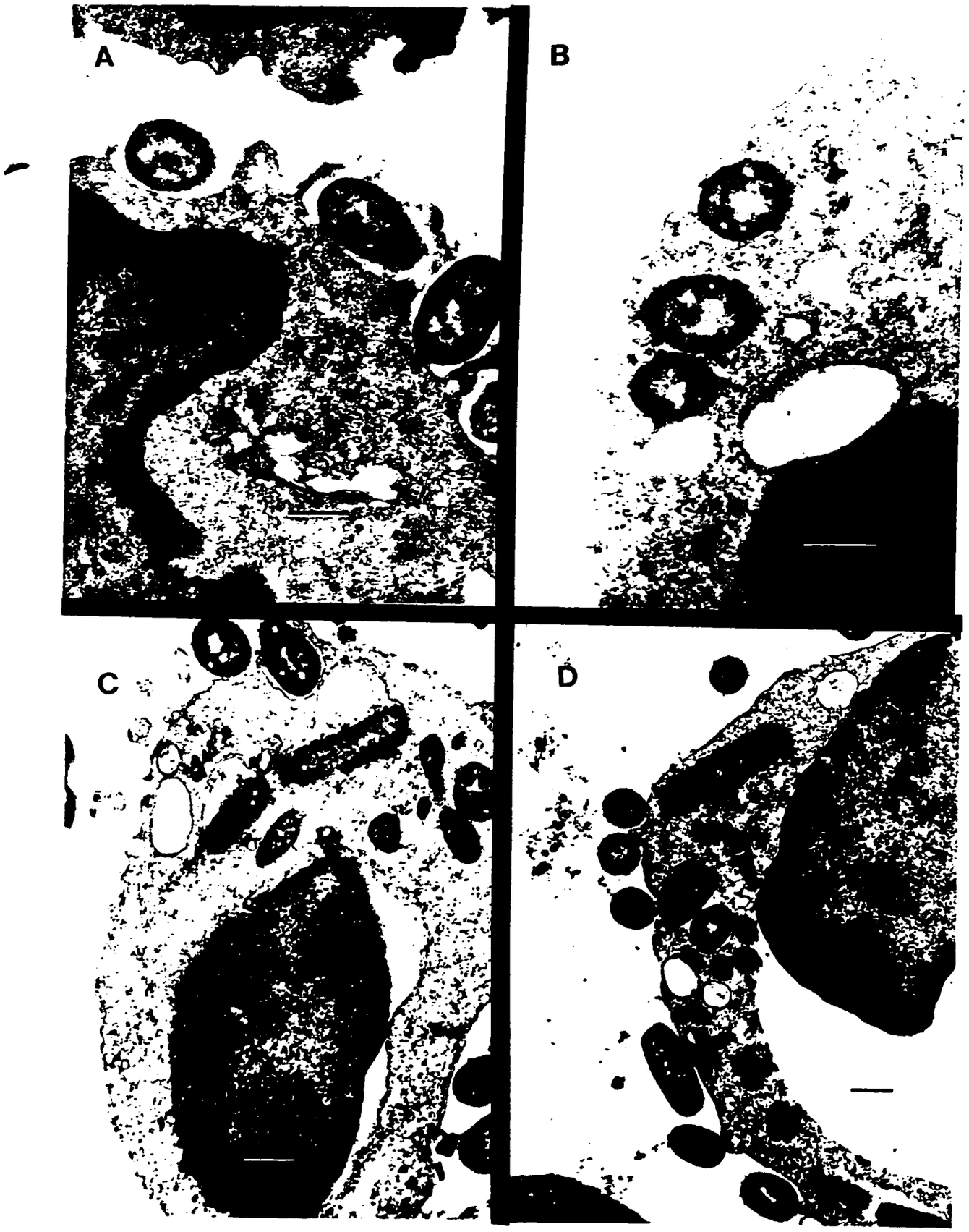

A
B
C
D

Fig. 8: Ultrastructural demonstration of *H. somnus* in bovine blood monocytes (BBM). *H. somnus* added to BBM in polypropylene tubes and incubated for 2 h; then cell pellets were washed two times to remove extracellular bacteria and added the macrophage medium containing gentamycin to control the extracellular bacteria. (A, B, C, and D micrographs indicating 24 h after infection with *H. somnus*) Undigested bacteria in vacuoles of BBM. (C) A higher number of bacteria in a necrotic BBM. Note a dividing bacterium. (D) A higher magnification of C. [Bar = 0.05μm]

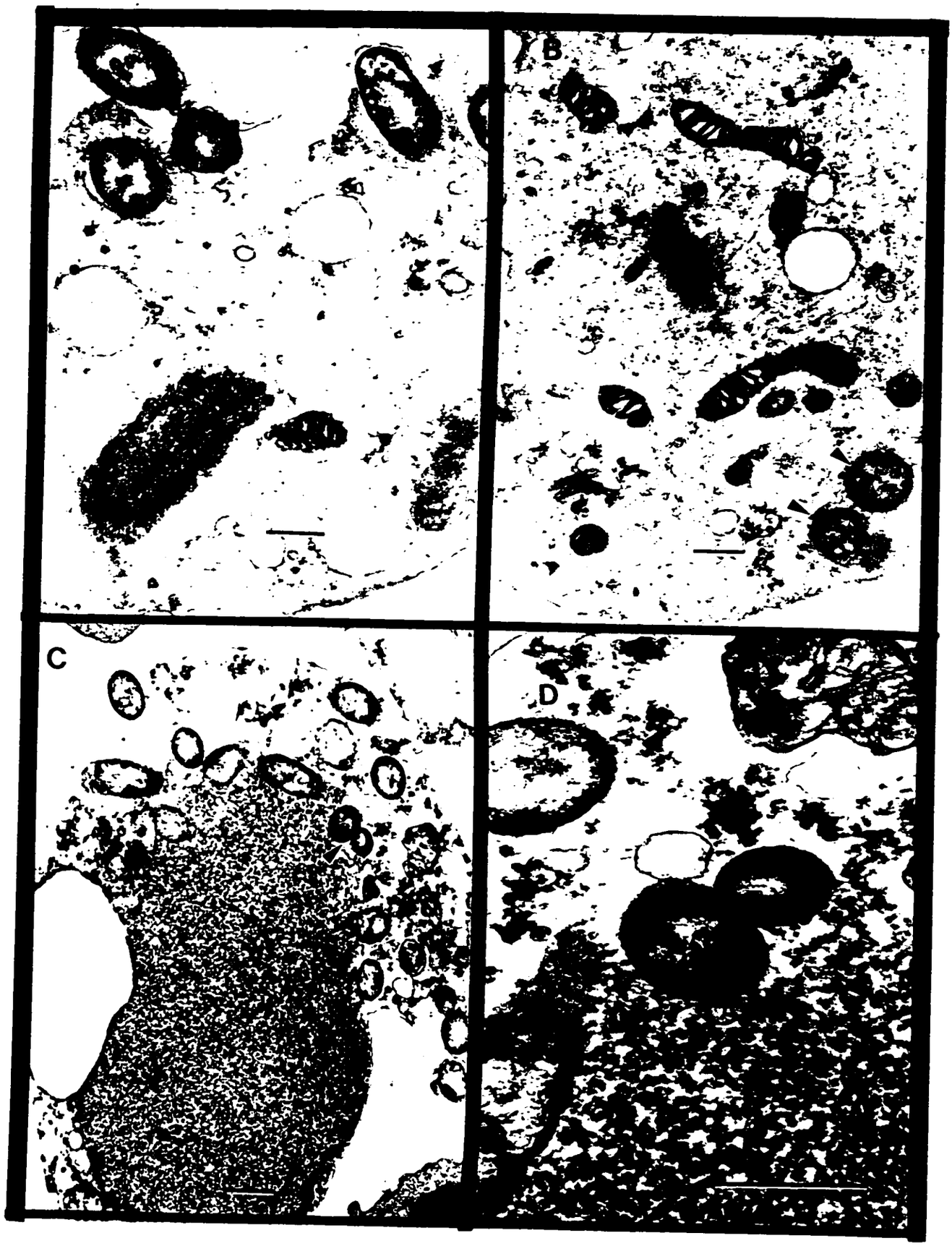

B
C
D

Fig. 9: Ultrastructural demonstration of *H. somnus* in BAM. *H. somnus* added to BAM in polypropylene tubes and incubated for 2 h; then cell pellets were washed two times to remove extracellular bacteria and added the macrophage medium containing gentamycin to control the extracellular bacteria. (A, and B: micrographs indicating 2 h after infection with *H. somnus*) (A) Some bacteria are internalised and some are surrounded by pseudopodia of alveolar macrophages. (B) A higher magnification of a membrane bound bacteria as seen in BBM. (C) 8 h after infection with *H. somnus*. Membrane bound undigested bacterium in a vacuole. [Bar(A) = 1μm, Bar(B) & (c) = 0.05μm]

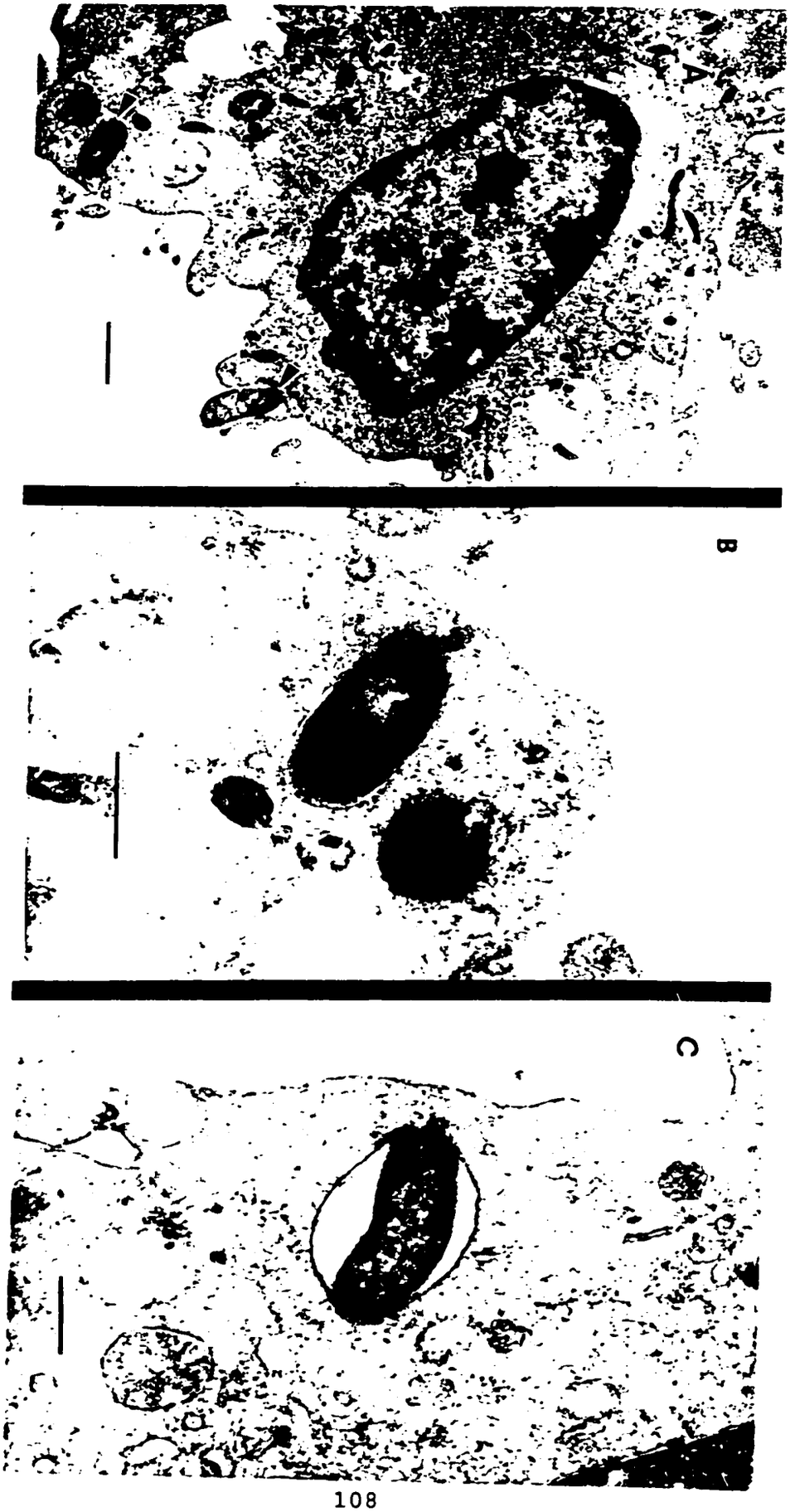

A
B
C

Fig. 10: ^{3}H uracil incorporation by bovine blood monocytes (BBM) infected with *H. somnus*. ^{3}H uracil was added to BBM infected with *H. somnus* and incubated for 12 h. Control samples contain either BBM or *H. somnus* alone. Back ground counts per minutes (CPM) was < 200. [B = bovine blood monocyte, s = *H. somnus*] The ratios refer to the number of bacteria per phagocyte. Each value shown is the mean and standard deviation. [B alone = BBM control; s1, s0.1, & s0.01 = *H. somnus* only controls contain bacteria according to test samples]

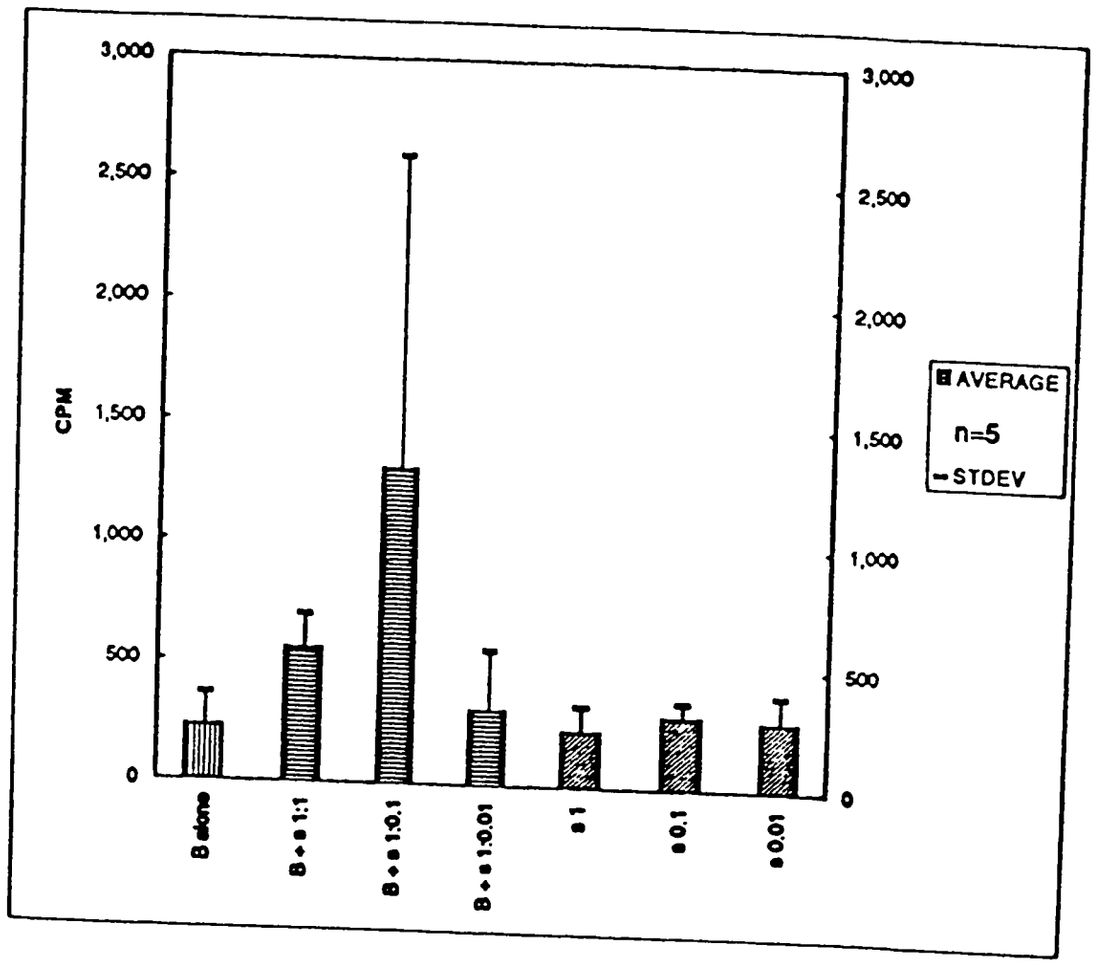

3,000
2,500
2,000
1,500
1,000
500
0
CPM
B alone
B + e 1:1
B + e 1:0.1
B + e 1:0.01
e 1
e 0.1
e 0.01
AVERAGE
n=5
STDEV

FIG. 11: ^{3}H uracil incorporation by bovine alveolar macrophages (BAM) infected with *H. somnus*. ^{3}H uracil was added to BAM infected with *H. somnus* and incubated for 12 h. Control samples contain either BAM or *H. somnus* alone. Back ground counts per minutes (CPM) was < 200. [A = bovine alveolar macrophages, s = *H. somnus*] The ratios refer to the number of bacteria per phagocyte. Each value shown is the mean and standard deviation. [A alone = BAM control; s1, s0.1, & s0.01 = *H. somnus* only controls contain bacteria according to test samples]

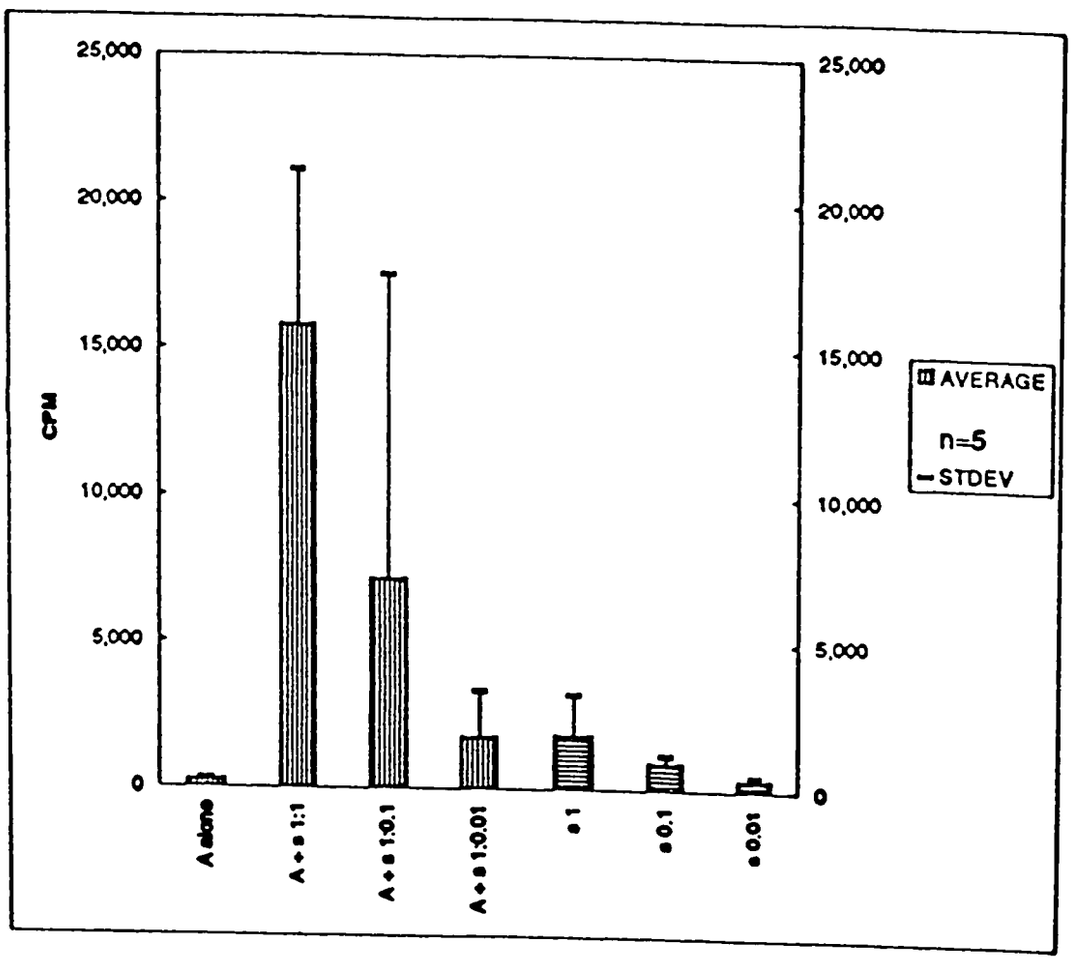

25,000
20,000
15,000
10,000
5,000
0
CPM
AVERAGE
n=5
STDEV
A alone
A + s 1:1
A + s 1:0.1
A + s 1:0.01
s 1
s 0.1
s 0.01

Fig. 12: ^{3}H uracil incorporation by bovine blood monocytes (BBM) infected with *H. somnus*. ^{3}H uracil was added to isolated BBM from cattle experimentally infected with *H. somnus*. No *in vitro* addition of *H. somnus* was made in these experiments. Back ground counts per minutes (CPM) was < 200. (12A) ^{3}H uracil incorporation by bovine blood monocytes (BBM) recovered from cattle experimentally infected with *H. somnus*. (12B) *H. somnus* isolation from BBM of same animals on same day of infection. Note the relationship between multiphasic pattern of bacteremia with high CPM (compare day 9 of 12A and 12B).

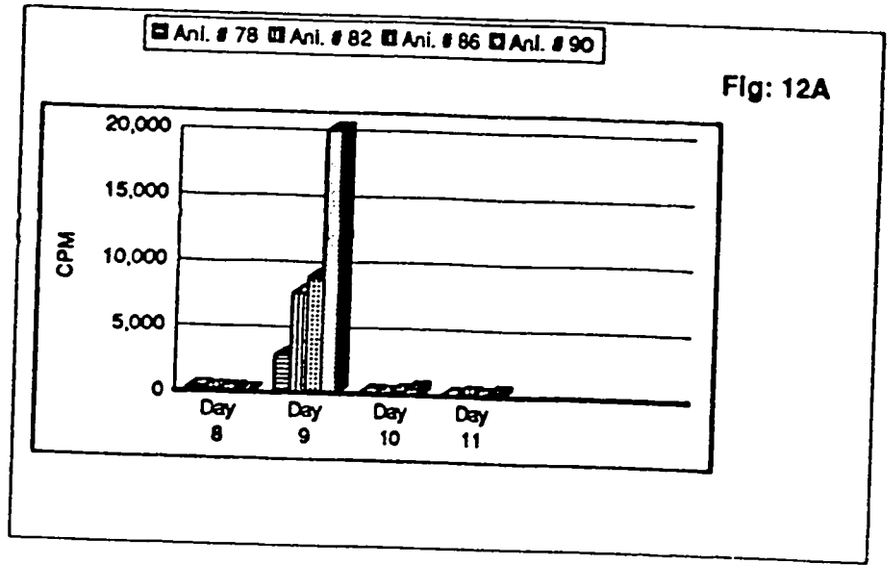


Fig: 12A

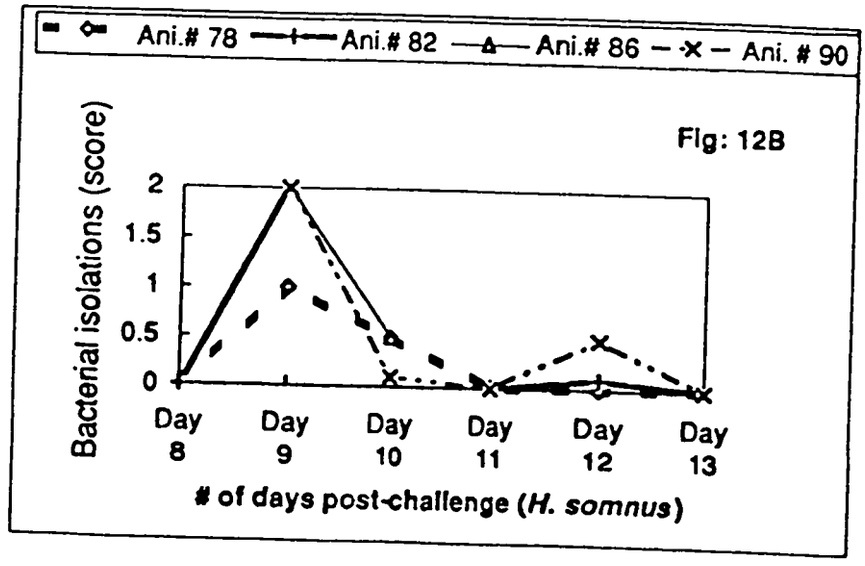


Fig: 12B

5. EFFECT OF *HAEMOPHILUS SOMNUS* ON NITRIC OXIDE PRODUCTION AND CHEMILUMINESCENCE RESPONSE OF BOVINE BLOOD MONOCYTES AND ALVEOLAR MACROPHAGES

ABSTRACT

Haemophilus somnus is able to survive and multiply in bovine blood monocytes (BBM) and alveolar macrophages (BAM) but the mechanisms used by *H. somnus* to evade killing mechanisms of bovine mononuclear phagocytes are not completely understood. To study the bactericidal ability of bovine mononuclear phagocytes following interaction with *H. somnus*, *in vitro* assay systems were developed to detect the luminol-dependent chemiluminescence response (LDCL) and nitric oxide (NO) production of BBM and BAM. Live logarithmically growing or stationary phase *H. somnus* rapidly inhibited the LDCL of BBM and BAM costimulated with opsonized *Staphylococcus aureus*. Inhibition of the LDCL response of BBM and BAM was not mediated by live *H. somnus* opsonized with hyperimmune serum or killed *H. somnus*. Recombinant BoIL-1β and *E. coli* LPS were better than rBoGM-CSF at inducing BBM and BAM to produce NO. BBM treated with rBoIL-1β and *E. coli* LPS produced less NO than did BAM treated with rBoIL-1β and *E. coli* LPS. *H. somnus* stimulated both BBM and BAM to produce NO. The ability of *H. somnus* to inhibit LDCL of both

BBM and BAM may be an important mechanism that contributes to the survival of the organism following ingestion by bovine mononuclear phagocytes.

INTRODUCTION

Haemophilus somnus is a Gram-negative, catalase-negative pathogen that causes a variety of clinical manifestations in cattle, collectively known as the *H. somnus* complex (Harris and Janzen, 1989). Furthermore, *H. somnus* has been shown to inhabit the reproductive and upper respiratory tract of healthy cattle (Corstvet *et al.*, 1973; Humphrey *et al.*, 1982b; Little, 1986). It has been reported that *H. somnus* is able to survive and multiply in bovine alveolar macrophages (BAM) and blood monocytes (BBM) (Lederer *et al.*, 1987). Since pathogenic and non-pathogenic strains of *H. somnus* can be isolated from the reproductive and respiratory tracts of clinically normal animals, it is not clear what host or microbial factors are responsible for this host-parasite relationship.

After exposure to a phagocytic stimulus, a very early manifestation of phagocytic cells is the generation of chemiluminescence (Allen and Loose, 1976). Bioluminescence in phagocytic systems is a nonspecific measurement of the respiratory burst, and may be due, at least in part, to the interaction of superoxide anion ($O_2^{\cdot}$) with the phagocytic stimuli (Babior, 1978). The chemical luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) has been used with *in vitro* phagocytic cell systems to enhance the sensitivity of monitoring oxygen-dependent hexose monophosphate shunt (HMPS) activity (Allen and Loose, 1976). Luminol interacts with the oxygen radicals produced during oxygen-dependent HMPS activity, resulting in the formation of an excited

aminophthalate anion. Upon relaxation to ground state, the aminophthalate anion produces light (Wilson *et al.*, 1978). The luminol dependent chemiluminescence (LDCL) assay is useful for evaluating phagocytic cell function (Allan, 1977; Wilson *et al.*, 1978; Stevens *et al.*, 1978; Easmon *et al.*, 1980).

Recently, the cytotoxic capacity of nitric oxide, produced by nitrogen oxidation of L-arginine, has been demonstrated for parasites (Adams *et al.*, 1990; James and Hibbs, 1990; Liew *et al.*, 1990; Green *et al.*, 1991; Mauel, *et al.*, 1991; Vincendeau and Daulouede, 1991; Li and Chadee, 1992). Macrophage cytotoxic activity correlates with the production of L-arginine-derived nitrite (NO_2^-), which is a stable degradation product of the freely diffusible, short-lived, and highly toxic effector molecule nitric oxide (Hibbs *et al.*, 1987; Hibbs *et al.*, 1987a; Stuehr and Nathan, 1989; Granger, *et al.*, 1990). The toxicity of nitric oxide (NO) is a result of the inhibition of selected iron-dependent enzymatic pathways involved in cellular respiration, energy production, and DNA synthesis of target cells (Hibbs *et al.*, 1987a; Drapier and Hibbs, 1988; Ignarro, 1991; Stuehr and Nathan, 1989).

The mechanisms involved with *H. somnus* survival in bovine mononuclear phagocytes are not fully understood. The objective of this study was to further characterize the microbial killing mechanisms of bovine mononuclear phagocytes following interaction with *H. somnus*.

MATERIALS AND METHODS

Bacteria

A pathogenic strain of *H. somnus* (HS25) originally isolated from the

pneumonic lung of a calf was obtained from Alberta Agriculture in Edmonton, Alberta was maintained in egg yolk, at -70C. Egg yolk stock culture was made by inoculating the pure cultures into 6-7 day old embryonated chicken eggs and incubating them at 37C until death of embryo occurred 1-3 days later. The egg yolks were then removed under sterile conditions, aliquoted, and stored at -70C. The isolate HS25 was selected for this study based on past research at V.I.D.O., including studies of interaction between polymorphonuclear leucocyte (PMN) and *H. somnus* (Pfeifer, 1992), and bovine animal model studies (Harland *et al.*, 1990). Before each experiment, a sample aliquot of egg yolk stock culture was thawed and spread onto a 5% sheep blood agar plate (PML Microbiologicals, Richmond, B.C.) and incubated for 36 h at 37C in an atmosphere containing 5% CO_2. A loopful of colonies was transferred to BHITT media [Brain Heart Infusion (Difco, Detroit, MI) supplemented with 1 µg/ml of 1% thiamine monophosphate (Sigma, St. Louis, MO), and 10 µg/ml of 10% Trizma base (Sigma, St. Louis, MO)], and incubated aerobically for 10 h at 37C on a shaker. As the final step, this broth culture was diluted to 1:4 with BHITT and incubated for 2 h until the optical density (OD) (660 nm) reached 0.4. One ml of this broth was found to contain approximately $1X10^9$ logarithmically growing *H. somnus*. Stationary phase *H. somnus* cultures were aerobically grown for 24 h at 37C in 10 ml of BHITT on a shaker. One ml of this broth contained approximately $1X10^9$ stationary phase *H. somnus*. The actual number of bacteria per ml was determined by plating 10-fold serial dilutions of the suspension in duplicate and counting colonies. This number was used to calculate the ratio of phagocytes to bacteria in all experiments. *H. somnus* was recovered and washed twice with cold (4C) Hanks balanced salt solution (HBSS) by centrifugation

before being used. Both logarithmically growing and stationary phase *H. somnus* were killed by heating at 65C for 30 min.

The culture of *Staphylococcus aureus* used was originally isolated from a case of bovine mastitis and was subsequently frozen at -70C in skim milk as a pure stock culture. A sample of stock culture was thawed and spread on a 5% sheep blood agar plate and incubated for 24 h at 37C in an atmosphere containing 5% CO_2. After incubation, a colony of *S. aureus* was selected, and incubated in 100 ml of Luria-Bertani (LB) -broth (Difco, Detroit, MI) that was grown aerobically for 18 h at 37C on a shaker. One ml of this seed culture was mixed with 100 ml of LB-Broth and incubated for 6 h at 37C on a shaker. Approximately $1X10^9$ CFU of *S. aureus* were in 1 ml of this broth.

Source of Bovine Mononuclear Phagocytes

Bovine blood monocytes were obtained from clinically normal, 6-12 month old beef calves. These animals were maintained at V.I.D.O. research facilities at the University of Saskatchewan. Bovine alveolar macrophages were obtained from abattoir specimens. Lung lavages were collected into 2 litre flasks with HBSS containing 1% antibiotic-antimycotic solution (Sigma Chemical Co. St. Louis, MO) (10,000 units of penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml in 0.9% sodium chloride) at the abattoir and flasks were kept on ice while being transported to the laboratory.

Isolation of Bovine Blood Monocytes

Heparinized (Organon Teknika, Toronto, Canada) blood was centrifuged for 5 min at 37C and 1000 g to separate the buffy coat from the platelet rich plasma. The buffy coat was removed and diluted with HBSS and layered on Ficoll-Paque (Pharmacia LKB, Biotechnology, Uppsala, Sweden) gradient to obtain mononuclear cell-rich population. The cells from the interface were removed and washed twice with HBSS. To detect NO production of macrophages infected with *H. somnus*, isolated peripheral blood mononuclear cells (PBMC) were suspended in macrophage SFM-medium (serum free medium) with glutamine and without phenol red (Gibco BRL, Life Technologies Inc. Grand Island, NY) and supplemented with 5% fetal bovine serum (heat inactivated) (Gibco BRL, Life Technologies Inc. Grand Island, NY), 10 nM Hepes (Gibco BRL, Life Technologies Inc. Grand Island, NY), and 2 g/l $NaHCO_3$. For the chemiluminescence assay, PBMC were suspended in HBSS supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Mononuclear cell viability was determined by trypan blue exclusion, and averaged 95%. PBMC suspensions contained between 4-10% monocytes as determined by microscopic examination of stained smears (Diff-Quick Fixative, Baxter Healthcare Corporation, IL).

Isolation of Bovine Alveolar Macrophages

Lavage fluid was filtered with a nylon strainer and centrifuged at 4C for 20 min and 800 g. Cell pellets were suspended in HBSS and layered on Ficoll-Paque gradient to obtain mononuclear cells. Mononuclear cells were removed in the interphase and washed twice with HBSS. For the NO assay to detect NO production

of macrophages infected with *H. somnus*, were suspended in macrophage SFM-medium with glutamine and without antibiotics, phenol red and supplemented with 5% fetal bovine serum, 10 nM hepes, and 2 g/l $NaHCO_3$. For the chemiluminescence assay, alveolar macrophages were suspended in HBSS supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Alveolar macrophage viability, determined by trypan blue exclusion, averaged 95%. Lung cell suspensions contained between 65%-85% macrophages, as determined by microscopic examination of stained smears (Diff-Quick Fixative, Baxter Healthcare Corporation, IL).

Recombinant Bovine Cytokines

Recombinant bovine IL-1β (rBoIL-1β) (American Cyanamid Co., Princeton, NJ) was produced in an *E. coli* expression system. The stock solution of 6.8 mg of rBoIL-1β/ml had a specific activity of $7.7X10^6$ IU/mg protein as determined by a murine thymocyte proliferation assay. There was 125 EU of endotoxin/mg of protein as determined by the limulus amoebocyte assay (Whittacker Corp., Walkersville, MD). The recombinant bovine granulocyte-macrophage colony stimulating factor (rBoGM-CSF) (American Cyanamid Co., Princeton, NJ) was produced in an *E. coli* expression system. Recombinant BoGM-CSF had a protein concentration of 2.7 mg/ml with an endotoxin content of 30.2 EU/mg of protein, and specific activity of $11.8X10^6$ U/mg (50% proliferation of neonatal bovine bone marrow). For the NO assay, 0.375 μg/ml rBoGM-CSF, 0.5 μg/ml rBoIL-1β and 0.5 μg/ml *E. coli* LPS (serotype 0111:B4) (Sigma Chemical Co. St. Louis, MO) were used to activate BBM and BAM.

Chemiluminescence Assay

This assay was conducted in 3.5 ml 55X12 mm tubes (Sarstedt, Germany). Each tube contained $4X10^6$ BBM or BAM, 700 µl of HBSS containing opsonized *S. aureus*, 200 µl of HBSS containing *H. somnus* and 15 µl of 5-amino-2,3-dihydro-1,4-phthalazinedion (luminol) (Sigma Chemical Co. St. Louis, MO). Both log-phase and stationary phase *H. somnus* were used in separate tubes. Three different ratios of phagocytes to *H. somnus* (1:1, 1:10, and 1:100) were used. Opsonized *H. somnus* (with 5% hyperimmunized serum for 30 min at 37C in a shaking water bath) and killed *H. somnus* were added into separate tubes. HBSS was substituted for *H. somnus* in the controls. Control samples contained bovine phagocytes with *H. somnus* without *S. aureus*. Chemiluminescence readings were measured on the Picolite Model 6500 luminometer (United Technologies Packard, Downers Grove, IL) every 3 min for 10 sec each over a period of 90 min. Constant stirring and a temperature of 37C were maintained throughout the entire assay. Duplicates were performed for each sample. The LDCL represented the total amount of light emitted over 90 min from exited oxygen species during bovine mononuclear phagocyte (BMP) respiratory burst. Bovine anti-*S. aureus* convalescent serum came from animals that had recovered from *S. aureus* mastitis. *H. somnus* hyperimmunized sera were prepared from cattle inoculated with HS25 several times.

$$\% \text{ Activity Remaining} = \frac{\text{(results of BMP with } H. somnus\text{)}}{\text{(results of BMP without } H. somnus\text{)}} \times 100$$

Nitrite (NO_2^-) Determination

Nitric oxide is highly unstable in solution and therefore cannot be readily assayed. However, NO is converted to the stable nitrite ion in aqueous solution, and NO production is routinely monitored by measuring supernatant nitrite concentration (Nicholas and Nason, 1957). Nitrite concentrations were measured using the Greiss reagent (Ding *et al.*, 1988). Briefly, 100 µl aliquot of culture supernatant was mixed with an equal volume of Greiss reagent (1% sulphanilamide, 0.1% naphtylethylene diamine dihydrochloride in 2.5% H_2PO_3) (Sigma Chemical Co. St. Louis, MO) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in an automated 96-channel microtitre plate reader (Bio-Rad Laboratories, model 3550, Richmond, CA). Nitrite concentration was calculated from a $NaNO_2$ standard curve (Fig. 1). The NO production assay was conducted in 96 well, flat bottom microtitre plates (Costar, Cambridge, MA). All microtitre wells contained $1X10^6$ (in 50 µl) BAM or BBM, and *H. somnus* (in 10 µl) and were incubated for 2 h at 37C in atmosphere containing 5% CO_2. Three different ratios of phagocytes to *H. somnus* (1:1, 1:10, and 1:100) were used. At the end of a 2 h incubation period, plates were washed twice with macrophage medium and 200 µl of macrophage medium containing 1 µg/ml Gentamycin was added to control the extracelluar *H. somnus* (Gibco BRL, Life Technologies Inc. Grand Island, NY). The supernatant was collected at 12, 24, 48, 72, and 96 h. BAM were incubated with 10% antibiotic-antimycotic solution (Sigma Chemical Co. St. Louis, MO) for 2 h at 4C to eliminate contaminants before beginning of NO assay. BAM or BBM were treated with recombinant bovine cytokines or *E. coli* LPS to detect the effect of treatments on NO production. At time 0 and 96 h, samples

from microtitre plates were cultured on 5% sheep blood agar plates in duplicate to confirm the survival of *H. somnus* in BBM and BAM and also to confirm the purity of *H. somnus* cultures.

Statistical Analysis

Analysis of variance (ANOVA) was executed using Minitab (Minitab for Windows, ver. 9.2, State College, PA.) in a Randomized Complete Block Design, to test the significance of the treatments. Statistical significance was assumed at $P \leq 0.05$. Treatment effect over time was considered as an additional factor in the design. Therefore, overall analyses were considered in a Split-plot design by considering time of observation as subplot. Mean comparisons were carried out using Lsd (least significant difference) test whenever the F test for treatment was significant at 5% level.

RESULTS

Chemiluminescent Response of BBM and BAM infected with *H. somnus*

The LDCL response of BBM was significantly reduced compared to the control, by either live logarithmically growing ($30.5 \pm 7.78\%$, $P<0.0001$) (Fig. 6) or stationary phase *H. somnus* ($54.5 \pm 2.12\%$, $P<0.0001$) (Fig. 7) when the phagocyte to *H. somnus* ratio was 1:100. The inhibitory effect seen with live *H. somnus* was completely absent when killed or opsonized (with hyperimmune serum) *H. somnus* was used. The LDCL response of BBM tended to increase when *H. somnus* was either killed or opsonized, reaching a peak level of 150-200% when BBM interacted with

opsonized *H. somnus* (Fig. 6 & 7). The LDCL response of BAM was significantly reduced by either live, logarithmically growing (14.25 ± 3.30%, P<0.001) (Fig. 8) or stationary phase *H. somnus* (49 ± 7.87%, P<0.006) (Fig. 9) when the phagocyte to *H. somnus* ratio was 1:100, as with BBM, the inhibitory effect of *H. somnus* on BAM was abrogated either by killing or opsonizing *H. somnus*. The control samples containing BBM or BAM with *H. somnus* had poor chemiluminescence response (data not shown).

NO Production by Cytokine Treated BBM and BAM

Recombinant BoIL-1β and *E. coli* LPS were better inducers of BBM and BAM to produce NO than rBo-GM-CSF (compare Figs. 2 and 3). Nitric oxide production by BBM treated with IL-1β or *E. coli* LPS at 48 h and 72 h (P<0.001) was significantly higher compared to controls at each time point (Fig. 2) whereas, NO production by BAM treated with IL-β or *E. coli* LPS was significantly higher at 24 h, 48 h, and 72 h (P<0.0001) (Fig. 3). Bovine alveolar macrophages treated with IL-1β or *E. coli* LPS produced significantly larger amounts of NO than BBM at 24 h, 48 h and 72 h (P<0.0001) (compare Figs. 2 and 3).

NO Production by BBM or BAM Infected with *H. somnus*

Nitric oxide production by BBM infected with *H. somnus* (at the ratio of 1:10 phagocytes to bacteria) was significantly higher at 12 h (P<0.001) whereas, NO production by BAM infected with *H. somnus* was significantly higher at 12 h (P<0.021), 24 h (P<0.005), and 48 h (P<0.006) compared to controls at each time point

(compare Figs. 4 and 5) (Nitric oxide values below detection limits of the assay are indicated as negative values). Bovine alveolar macrophages infected with *H. somnus* produced relatively greater amounts of NO than did BBM infected with *H. somnus* between 24 - 96 h period but this difference was not significant ($P<0.315$) (compare Figs. 4 and 5). BBM or BAM infected with opsonized (with 5% hyperimmune serum) *H. somnus* produced the same amount of NO as with unopsonized live bacteria (data not shown).

DISCUSSION

The exact sequences of events during phagocytosis, particularly with respect to the clinical interpretation of chemiluminescence assays, remains unclear. However, several authors have attempted to correlate chemiluminescence response with phagocytic and bactericidal activity (Johnston *et al.*, 1975; Grebner *et al.*, 1977; Welch, 1980; Meuwissen *et al.*, 1982). Generally, it is thought that luminol is dioxygenated to yield aminophthalate anions and subsequent electronically excited carbonyl chromophors which, upon relaxation to ground state, produce light (Allen, 1979). The process is subject to complex interactions between activating particles and cellular receptors. The LDCL assay was used to describe oxidative burst of bovine mononuclear phagocytes following interaction with *H. somnus*.

Although *H. somnus* is a catalase negative organism, Sample and Czuprynski (1991) demonstrated that *H. somnus* is capable of removing H_2O_2 from an aqueous solution in an energy dependent manner that is strictly cell associated. Hydrogen peroxide removal requires viable *H. somnus*. Reduction of LDCL activity of BBM and

BAM infected with *H. somnus* could be partly explained by H_2O_2 removal by viable *H. somnus*. Moreover, abrogation of reduced LDCL activity of BBM or BAM with heat killed *H. somnus* correlates with the observation of Sample and Czuprynski (1991) that viable *H. somnus* were required to remove H_2O_2. It is also clear that *H. somnus* was not a poor stimulus for bovine mononuclear phagocytes but that the organism interfered with the oxidative burst, since heat killed or opsonized *H. somnus* increased the LDCL activity of BBM or BAM. It is possible that inhibition of LDCL activity, in conjunction with suppression of mononuclear phagocytic functions, may be important virulence attributes which allow *H. somnus* to survive the oxidative burst of bovine mononuclear phagocytes.

Infection of BBM or BAM with *H. somnus* stimulated the production of NO. *H. somnus* was able to survive intracellularly in either BBM or BAM for 96 h while phagocytes were actively producing NO (data not shown); NO is not effective bactericidal mechanism for *H. somnus*.

Fig. 1: Nitric oxide standard curve. Relationship between optical density and NO_2^- concentration. Serial dilutions of sodium nitrite dissolved in MEM (minimum essential medium) containing 10% fetal bovine serum were used for this standard curve.

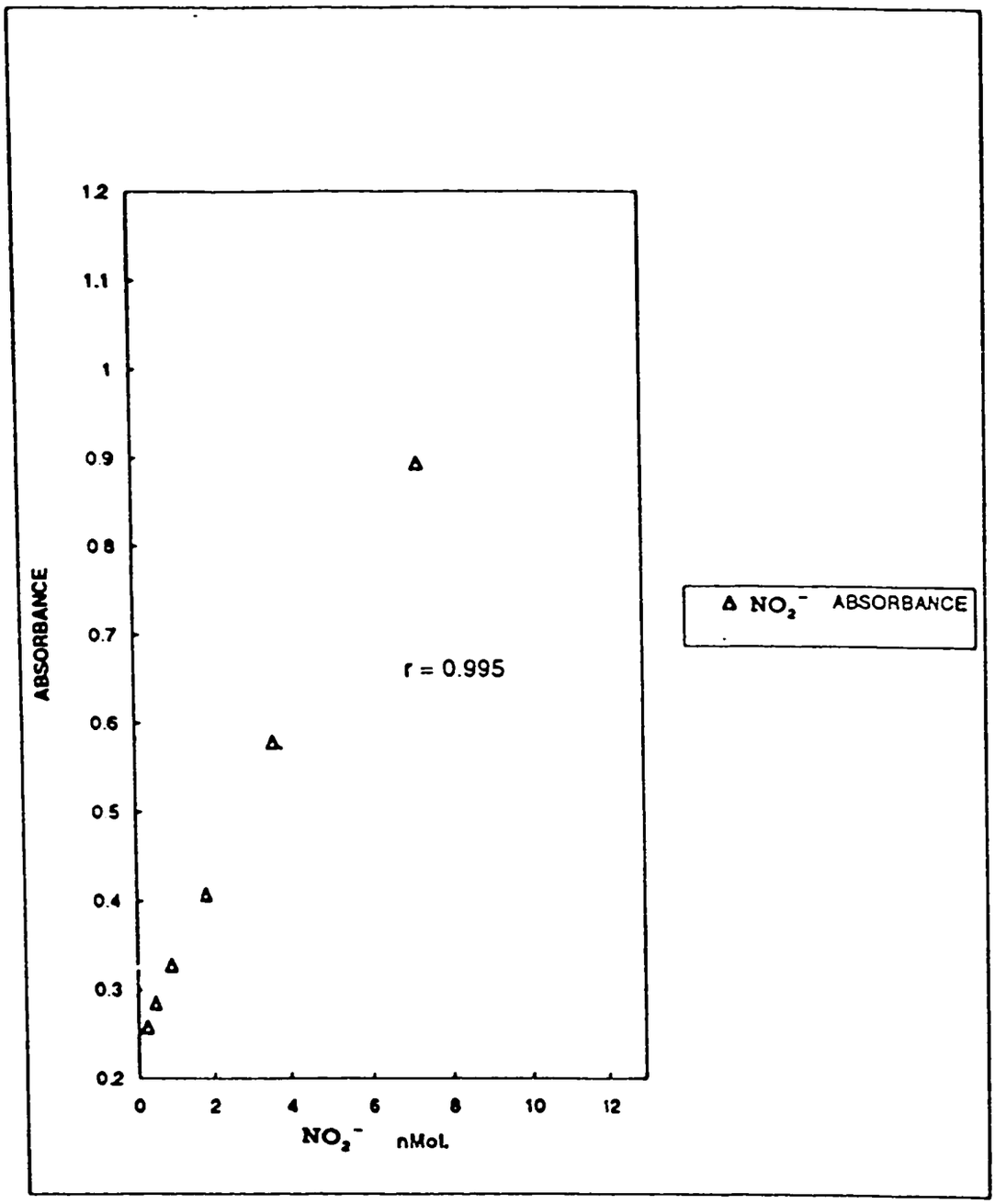

1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
ABSORBANCE
r = 0.995
0
2
4
6
8
10
12
NO_2^- nMol.
Δ NO_2^- ABSORBANCE

Fig. 2: Production of nitric oxide by bovine blood monocytes (BBM) treated with various recombinant bovine cytokines. BBM treated with various recombinant cytokines or *E. coli* LPS and incubated up to 72 h; culture supernatants were tested for NO_2^- concentration. (n=3) Each value shown is mean and standard deviation.

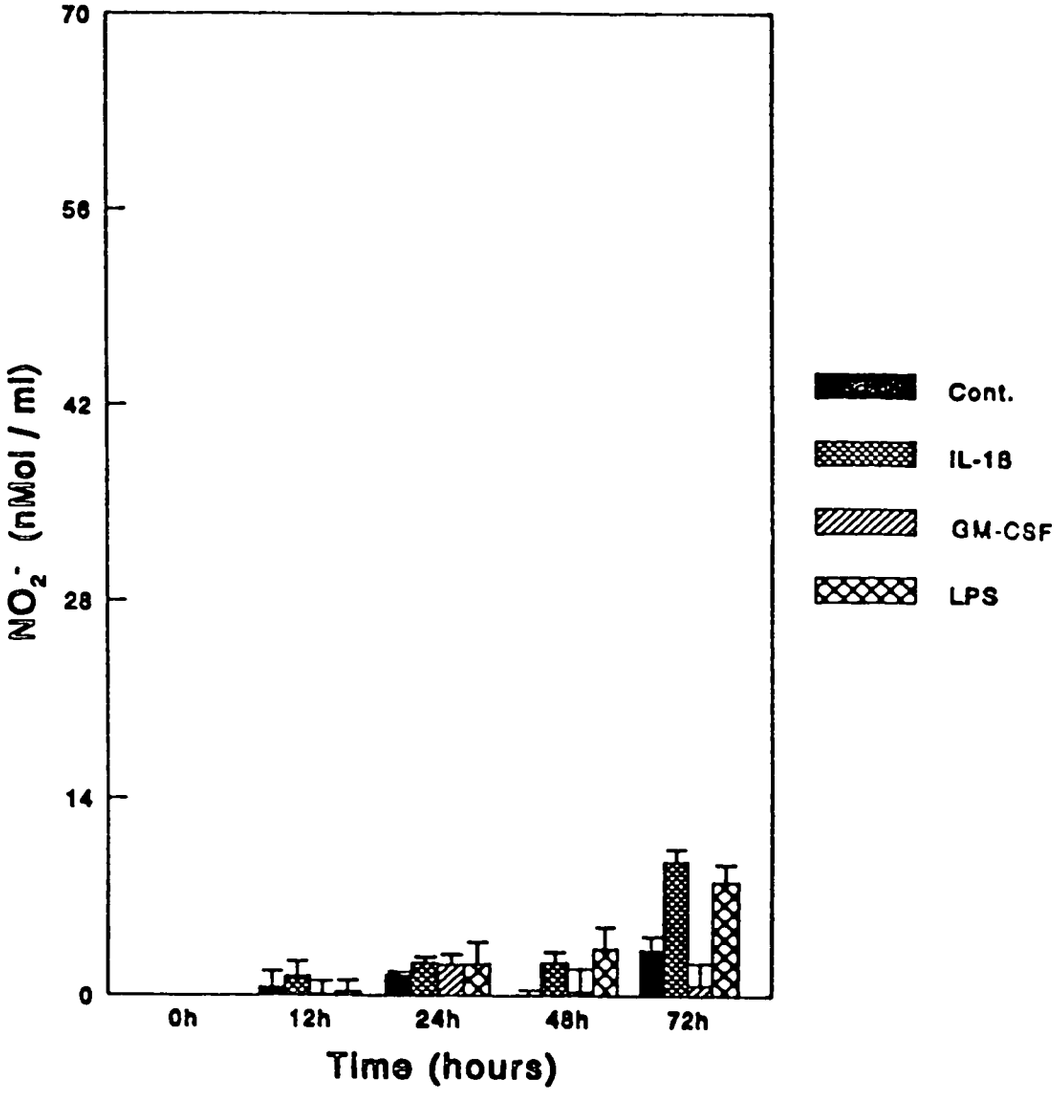

70
56
42
28
14
0
NO_2^- (nMol / ml)
0h
12h
24h
48h
72h
Time (hours)
Cont.
IL-1B
GM-CSF
LPS

Fig. 3: Production of nitric oxide by bovine alveolar macrophages (BAM) treated with various recombinant bovine cytokines. BAM treated with various recombinant cytokines or *E. coli* LPS and incubated up to 72 h; culture supernatants were tested for NO_2^- concentration. (n=3) Each value shown is mean and standard deviation.

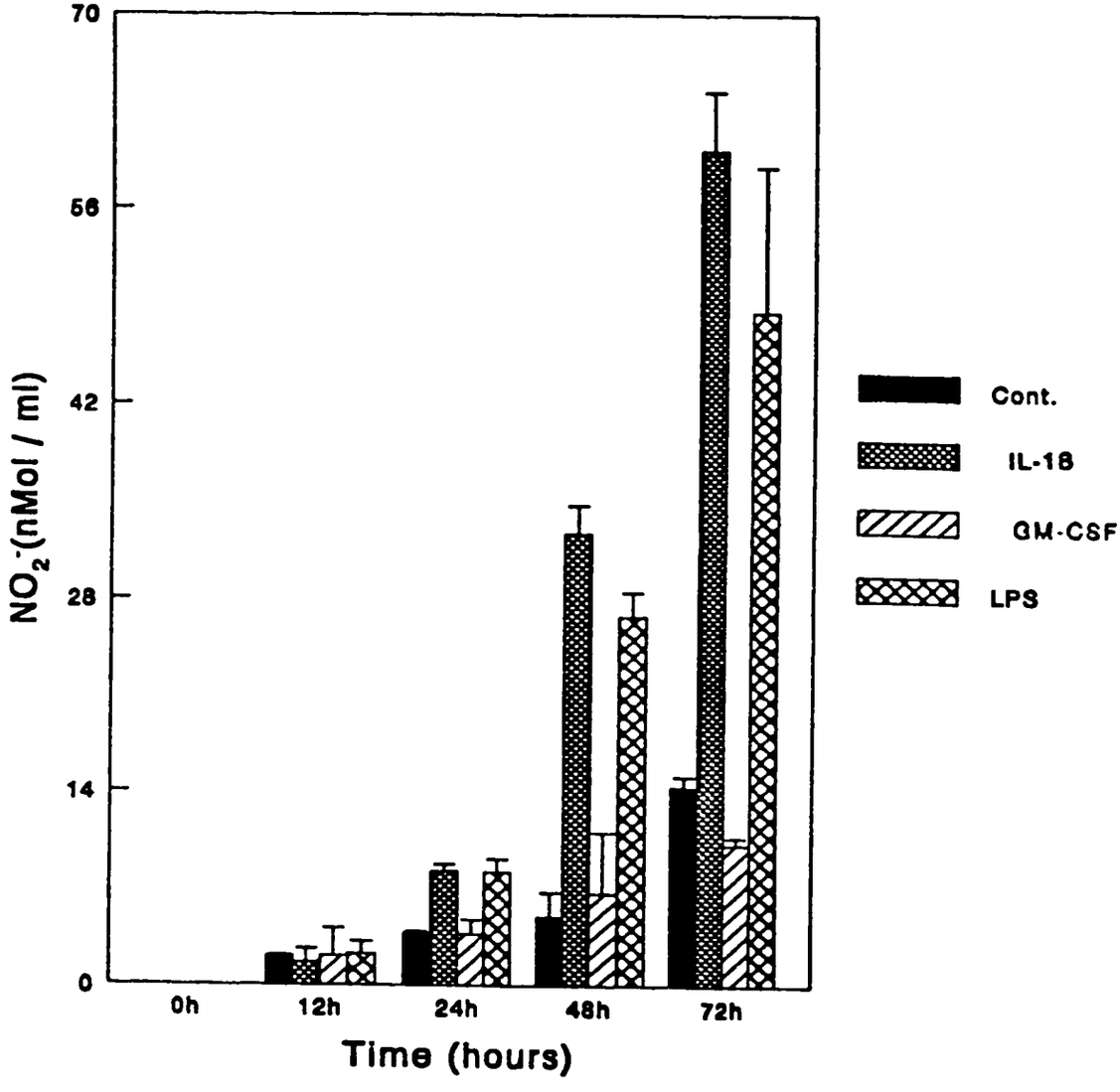

70
56
42
28
14
0
NO_2^-(nMol / ml)
0h
12h
24h
48h
72h
Time (hours)
Cont.
IL-1β
GM-CSF
LPS

Fig. 4: Production of nitric oxide by bovine blood monocytes (BBM) infected with *H. somnus*. BBM infected with *H. somnus* and incubated up to 96 h; culture supernatants were tested for NO_2^- concentration. (n=3) Each value shown is mean and standard deviation. (L-som = live *H. somnus*, K-som = killed *H. somnus*) (1:1, 1:10, & 1:100 = ratio of phagocytes to *H. somnus*)

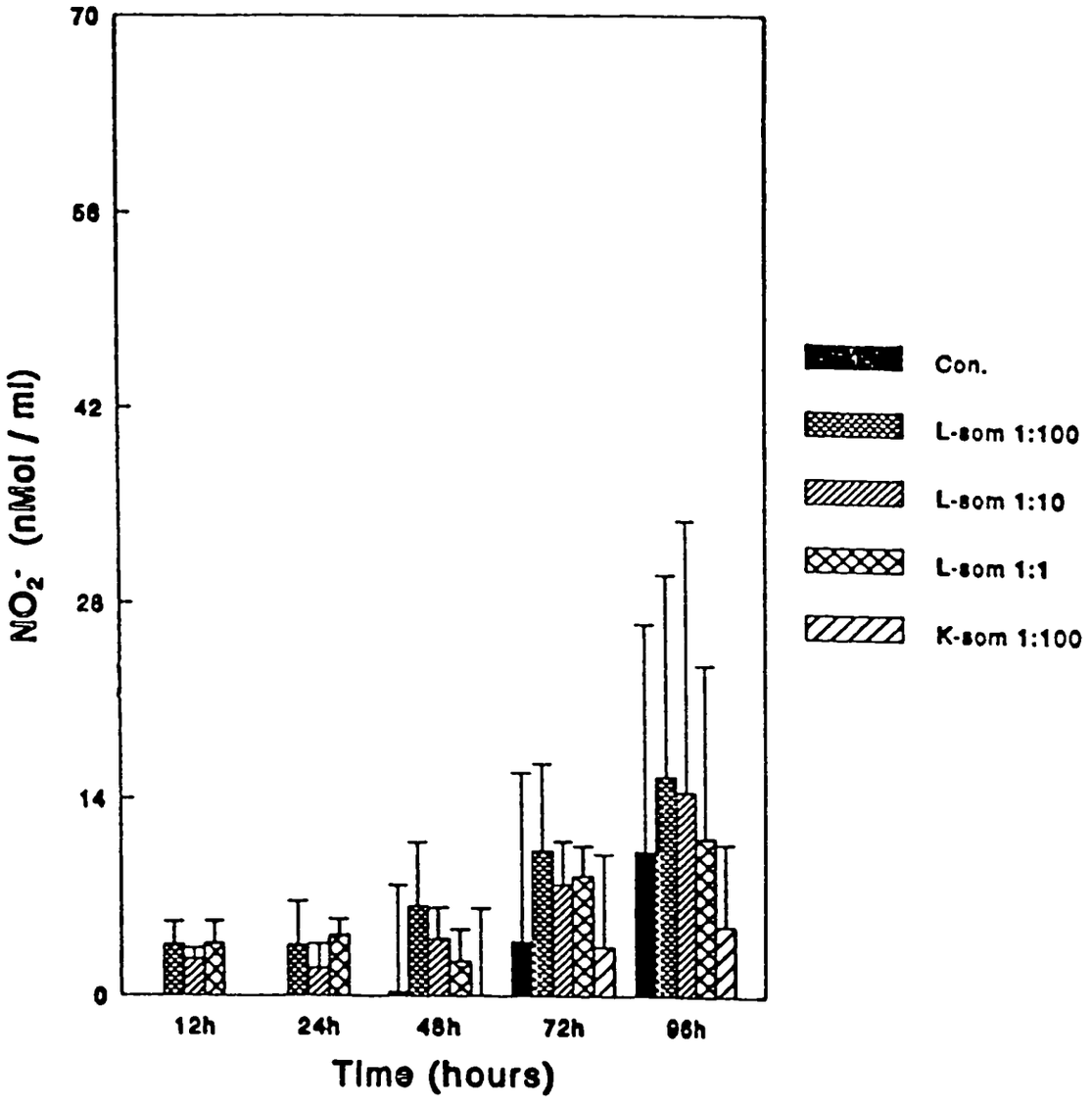

70
56
42
28
14
0
NO_2^- (nMol / ml)
12h
24h
48h
72h
96h
Time (hours)
Con.
L-som 1:100
L-som 1:10
L-som 1:1
K-som 1:100

Fig. 5: Production of nitric oxide by bovine alveolar macrophages (BAM) infected with *H. somnus*. BAM infected with *H. somnus* and incubated up to 96 h; culture supernatants were tested for NO_2^- concentration. (n=3) Each value shown is mean and standard deviation. (L-som = live *H. somnus*, K-som = killed *H. somnus*) (1:1, 1:10, & 1:100 = ratio of phagocytes to *H. somnus*)

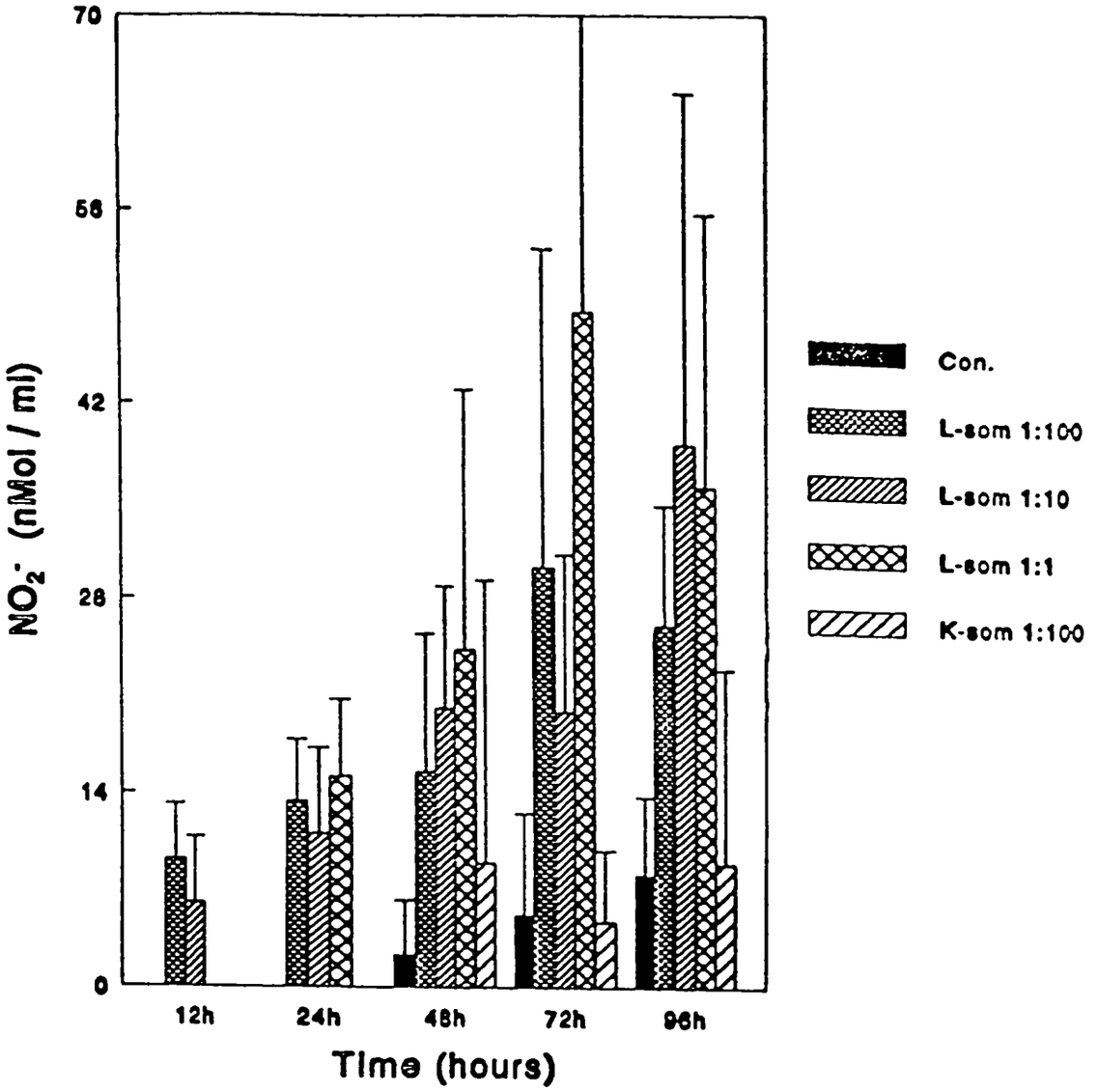

70
56
42
28
14
0
NO_2^- (nMol / ml)
12h
24h
48h
72h
96h
Time (hours)
Con.
L-som 1:100
L-som 1:10
L-som 1:1
K-som 1:100

Fig. 6: Luminol-dependent chemiluminescence (LDCL) response of bovine blood monocytes (BBM) with the interaction of logarithmically growing *H. somnus*. Live log phase *H. somnus* rapidly inhibited the LDCL response of BBM costimulated with opsonized *S. aureus*. Inhibition of the LDCL response of BBM was not mediated by live logarithmically growing *H. somnus* opsonized with hyperimmune serum or killed *H. somnus*. [log = logarithmically growing phase of *H. somnus*] (n=4) Each value shown is mean and standard deviation. (live, opsonized, & killed = live, opsonized or killed logarithmically growing phase of *H. somnus*; 1:10, 1:100, & 1:1000 = ratio of phagocytes to *H. somnus*)

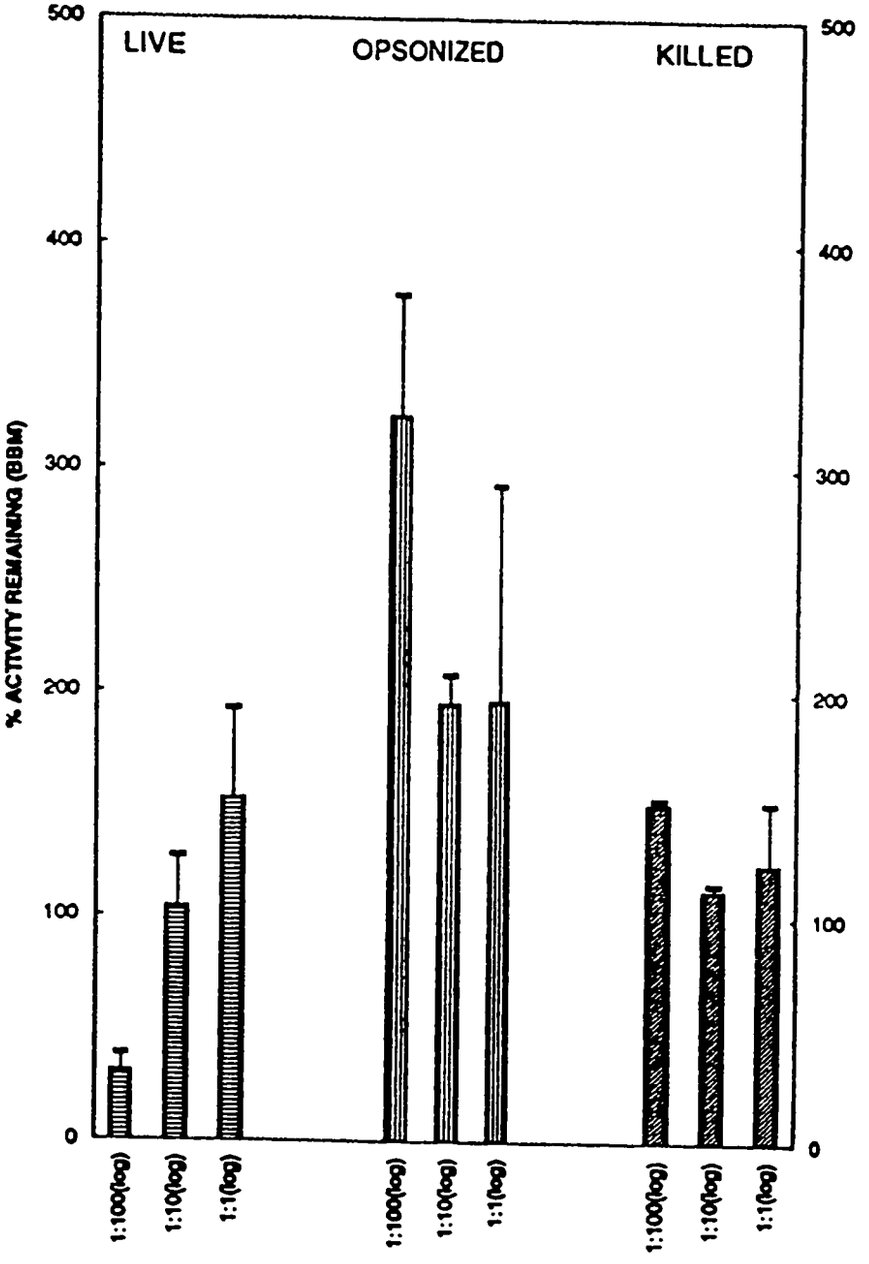

LIVE
OPSONIZED
KILLED
% ACTIVITY REMAINING (BBM)
500
400
300
200
100
0
1:100(log)
1:10(log)
1:1(log)
1:100(log)
1:10(log)
1:1(log)
1:100(log)
1:10(log)
1:1(log)

Fig. 7: Luminol-dependent chemiluminescence (LDCL) response of bovine blood monocytes (BBM) with the interaction of stationary phase *H. somnus*. Live stationary phase *H. somnus* rapidly inhibited the LDCL response of BBM costimulated with opsonized *S. aureus*. Inhibition of the LDCL response of BBM was not mediated by live stationary *H. somnus* opsonized with hyperimmune serum or killed *H. somnus*. Each value shown is mean and standard deviation. [st = stationary phase *H. somnus*] (n=4) (live, opsonized, & killed = live, opsonized or killed stationary phase of *H. somnus*; 1:10, 1:100, & 1:1000 = ratio of phagocytes to *H. somnus*)

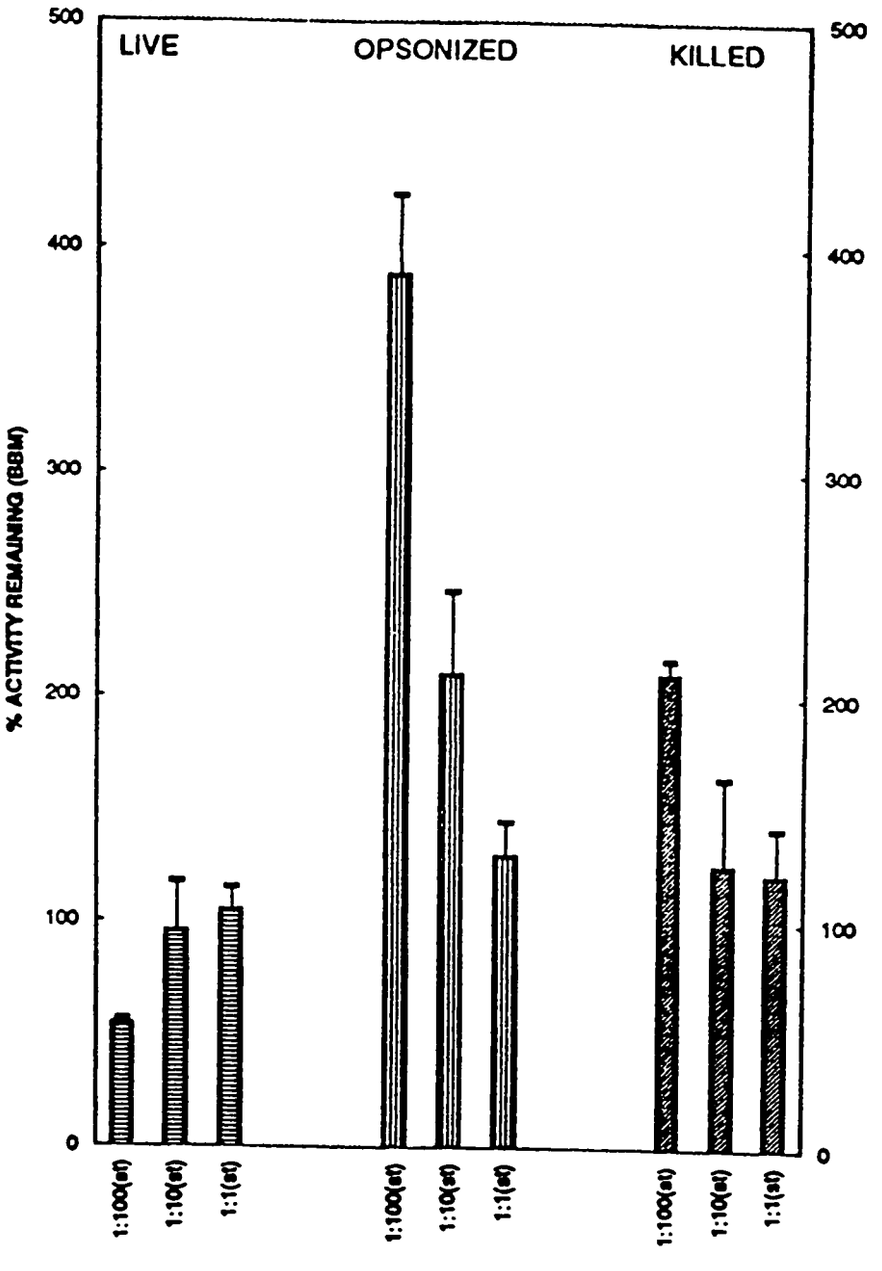

LIVE
OPSONIZED
KILLED
% ACTIVITY REMAINING (BBM)
500
400
300
200
100
0
1:100(st)
1:10(st)
1:1(st)

Fig. 8: Luminol-dependent chemiluminescence (LDCL) response of bovine alveolar macrophages (BAM) with the interaction of logarithmically growing *H. somnus*. Live log phase *H. somnus* rapidly inhibited the LDCL response of BAM costimulated with opsonized *S. aureus*. Inhibition of the LDCL response of BAM was not mediated by live logarithmically growing *H. somnus* opsonized with hyperimmune serum or killed *H. somnus*. [log = logarithmically growing phase of *H. somnus*] (n=4) Each value shown is mean and standard deviation. (n=4) (live, opsonized, & killed = live, opsonized or killed logarithmically growing phase of *H. somnus*; 1:0.1, 1:1, 1:10, & 1:100 = ratio of phagocytes to *H. somnus*)

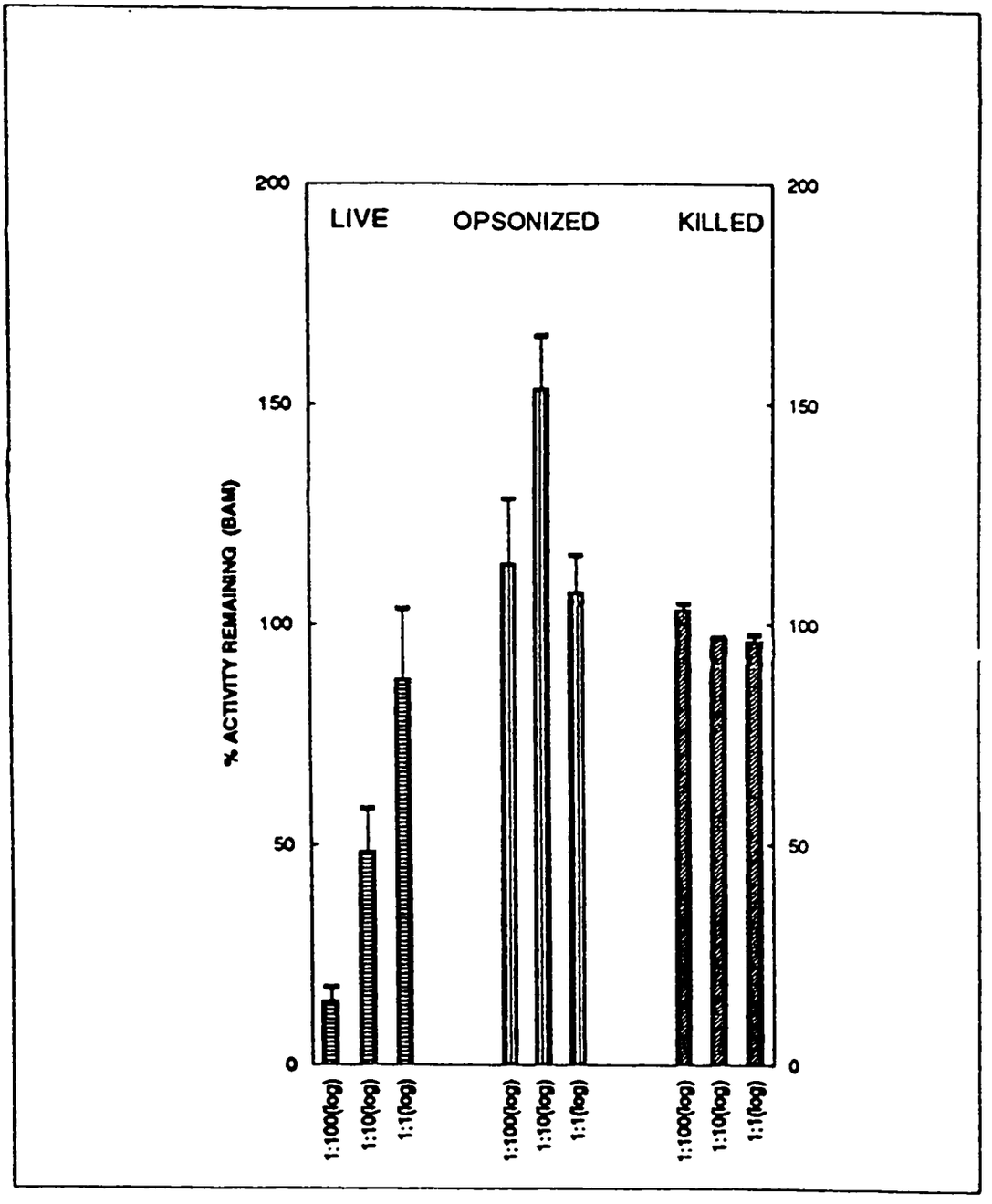
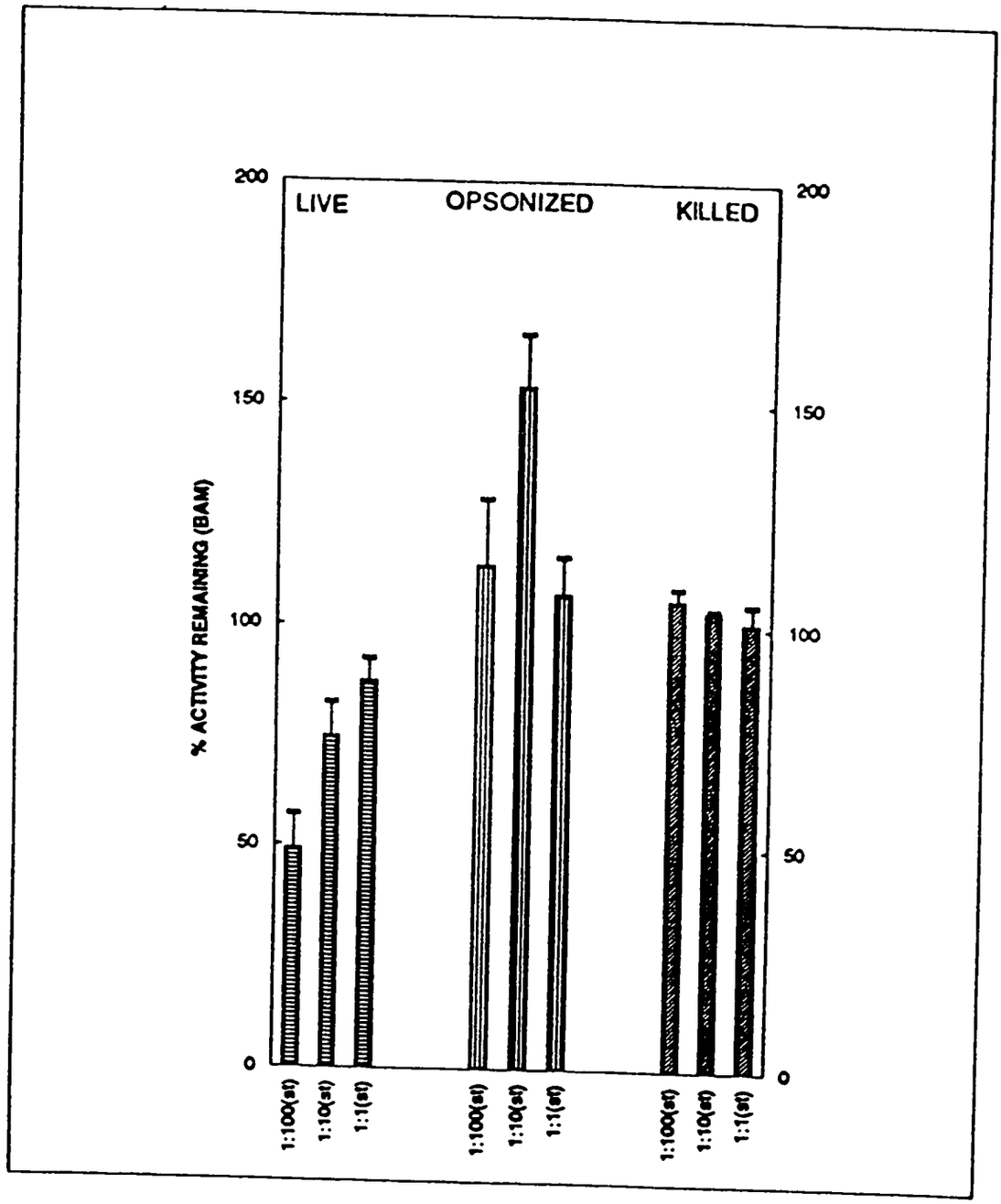

LIVE
OPSONIZED
KILLED
% ACTIVITY REMAINING (BAM)
200
150
100
50
0
1:100(log)
1:10(log)
1:1(log)

Fig. 9: Luminol-dependent chemiluminescence (LDCL) response of bovine alveolar macrophages (BAM) with the interaction of stationary phase *H. somnus*. Live stationary phase *H. somnus* rapidly inhibited the LDCL response of BAM costimulated with opsonized *S. aureus*. Inhibition of the LDCL response of BAM was not mediated by live stationary phase *H. somnus* opsonized with hyperimmune serum or killed *H. somnus*. [log = logarithmically growing phase of *H. somnus*] (n=4) Each value shown is mean and standard deviation. (live, opsonized, & killed = live, opsonized or killed stationary phase of *H. somnus*; 1:0.1, 1:1, 1:10, & 1:100 = ratio of phagocytes to *H. somnus*)


LIVE
OPSONIZED
KILLED
% ACTIVITY REMAINING (BAM)
200
150
100
50
0
1:100(st)
1:10(st)
1:1(st)
1:100(st)
1:10(st)
1:1(st)
1:100(st)
1:10(st)
1:1(st)

6. PRODUCTION OF BOVINE TUMOR NECROSIS FACTOR, INTERFERON-γ, AND INTERLEUKIN-6 FOLLOWING THE INTERACTION OF BOVINE LEUCOCYTES WITH *HAEMOPHILUS SOMNUS*

ABSTRACT

The pathogenesis of *Haemophilus somnus* infection in cattle is not fully understood. To determine the bovine blood cytokine response with the interaction of *H. somnus*, a whole blood culture assay system was developed. Blood samples from healthy calves were incubated with or without *H. somnus* for varying times up to 24 h. No detectable tumor necrosis factor (TNF) was found regardless of whether or not live *H. somnus* was present. When blood cultures were stimulated live *H. somnus*, interferon-γ (IFN-γ) and interleukin-6 (IL-6) became detectable at 8 h and were maintained at that level up for 24 h whereas, when simulated with heat killed *H. somnus*, IFN-γ or IL-6 were only detectable at 24 h. No detectable level of TNF, IFN-γ or IL-6 was found in serum of cattle experimentally infected with *H. somnus* between 0 and 7 days post-infection. Following *in vitro* addition of *H. somnus* into whole blood cultures prepared from cattle experimentally infected with *H. somnus*; TNF production was not detectable during the 24 h incubation period while IFN-γ was detected. The *ex vivo* IFN-γ response to *H. somnus* in whole blood cultures was temporally inhibited

in calves at day 2 post-infection, the time when most deaths resulted from infection, and recovered from day 3 on, with the maximal response seen on day 7 post-infection. The course of IL-6 production followed a slightly different pattern from that of IFN-γ, in that the IL-6 response was inhibited in calves on days 1 and 2 post-infection, was maximal on day 3, and then waned but maintained at a substantial level throughout the experiment. Intracellular survival and multiplication of *H. somnus* in bovine phagocytes could be responsible for these cytokine profiles.

INTRODUCTION

Tumor necrosis factor-α (TNF-α) is a well defined cytokine produced by mononuclear phagocytes and is responsible for the induction of both inflammatory and immune responses (Burchett *et al.*, 1988). TNF-α is a 17 kilodalton cytokine released primarily from monocytes and macrophages in response to a wide variety of inflammatory stimuli. At low concentrations, TNF-α contributes to homeostasis and host defence mechanisms, whereas at high concentrations it mediates various pathological conditions (Tracey *et al.*, 1989; Beutler 1990). TNF-α has been shown to mediate a wide variety of biological activities that play an important role in both the severity of and recovery from many bacterial, parasitic, and viral infections (Le and Vilcek, 1987; Rosenblum and Donato, 1989). These activities include induction of f147ever, direct activation of neutrophils, T cells, and macrophages; direct tumor cell cytotoxicity, cachexia, induction of other mediators (e.g., interleukin 1 [IL-1], and interleukin-6 [IL-6] and TNF itself) (Le and Vilcek, 1987; Rosenblum and Donato, 1989). Infusion of TNF-α induces cardiovascular shock, haemorrhagic necrosis in

tissues, and metabolic derangements similar to both experimental endotoxemia and clinical septic shock (Tracey *et al.*, 1986; Tracey *et al.*, 1987) whereas prior treatment with antibodies against TNF-α protects animals against the lethal effects of endotoxemia (Beutler *et al.*, 1985; Tracey *et al.*, 1986; Mathison *et al.*, 1988; Johnson *et al.*, 1989). Little is known about TNF-α production, or its biological activities, in ruminant species (Adams and Czuprynski, 1990).

Interferon-γ (IFN-γ) is a product of activated T lymphocytes and natural killer cells with immunomodulatory and antiviral properties (Sen and Lengyel, 1992). This cytokine is a potent inducer of activation and differentiation of mononuclear phagocytes (Espinoza-Delgado *et al.*, 1992; Varesio *et al.*, 1993), manifested by an increase in class II antigens (Kelley *et al.*, 1984; Littman *et al.*, 1989), toxic oxygen derivatives and nitric oxide production (Ding *et al.*, 1988; Cox *et al.*, 1992; Melillo *et al.*, 1993), regulation of cytokine production (Philip and Epstein, 1986; Arai *et al.*, 1990; Sen and Lengyel, 1992; Cox *et al.*, 1992; Melillo *et al.*, 1993), and tumoricidal activity (Cox *et al.*, 1992; Varesio *et al.*, 1993). IFN-γ has been shown in animal models to modulate macrophage tumoricidal and microbicidal activity against both intracellular and extracellular parasites (Pace *et al.*, 1983; Schreiber, 1984; Gazzinelli *et al.*, 1992; Oswald *et al.*, 1992), and B and T cell responses to antigens (Nakamura *et al.*, 1984; Frasca *et al.*, 1985).

Interleukin-6 is produced by T cells, fibroblasts, macrophages, and peripheral blood mononuclear cells (Van Snick *et al.*, 1987; Suda *et al.*, 1988; Wong *et al.*, 1988; Shabo *et al.*, 1988; Everson *et al.*, 1989; Qin, 1989; Snyers *et al.*, 1989; Van Snick, 1990). It has been reported that IL-6 is another important monocyte derived product

mediating the host defence against infectious agents (Gauldie *et al.*, 1987; May *et al.*, 1988; Tosato *et al.*, 1988). IL-6 enhances immune function by amplifying T-cell proliferation and cytotoxic T-cell differentiation and promoting B-cell proliferation and differentiation (Muraguchi *et al.*, 1988; Tosato and Pike, 1988; Kishimoto *et al.*, 1989). IL-6 is also a principal stimulus for the induction of acute-phase proteins from hepatocytes in both *in vitro* and *in vivo* studies (Gauldie *et al.*, 1987; May *et al.*, 1988; Castell *et al.*, 1988; Ramadori *et al.*, 1988; Andus *et al.*, 1988; Geiger *et al.*, 1988; Marinkovic *et al.*, 1989). IL-6 has been implicated in the pathophysiology of injury and infection (Castell *et al.*, 1989; Hedges, 1991). IL-6 can be found in circulating blood of patients with acute endotoxemia, acute infections, meningococcal sepsis and burns (Waage *et al.*, 1989; Schluter *et al.*, 1991). Administration of IL-6 induces some of the characteristic physiological derangements associated with injury including fever and counterbalances the effect of sepsis by the induction of acute phase responses (Perlmutter, *et al.*, 1986; Castell *et al.*, 1989).

It has been recently suggested that *ex vivo* whole-blood culture systems for evaluating cytokine production closely resemble *in vivo* conditions (Kato *et al.*, 1990; Yachie *et al.*, 1990; Yachie *et al.*, 1992) compared to use of isolated mononuclear cells, which are usually stimulated by various nonspecific simulators (Dike and Farmer, 1988; Yachie *et al.*, 1990). Cultures using whole blood appear to provide an ideal system to estimate the cellular response to stimulating agents *in vitro* (Yachie *et al.*, 1992). The objective of this study was to detect the endogenous TNF, IFN-γ, and IL-6 response to *H. somnus* infection.

MATERIALS AND METHODS

Bacteria

A pathogenic strain of *H. somnus* (HS25) originally isolated from the pneumonic lung of a calf was obtained from Alberta Agriculture in Edmonton, Alberta was maintained in egg yolk at -70C. Egg yolk stock culture was made by inoculating the pure cultures into 6-7 day old embryonated chicken eggs and incubating them at 37C until death of embryo occurred 1-3 days later. The egg yolks were then removed under sterile conditions, aliquoted, and stored at -70C. The isolate HS25 was selected for this study based on past research at V.I.D.O., including studies of interaction between polymorphonuclear leucocyte (PMN) and *H. somnus* (Pfeifer, 1992), and bovine animal model studies (Harland *et al.*, 1990). Before each experiment, an aliquot of egg yolk stock culture was thawed and spread onto a 5% sheep blood agar plate (PML Microbiologicals, Richmond, B.C.) and incubated for 36 h at 37C in an atmosphere containing 5% CO_2. A loopful of colonies was transferred to media BHITT [Brain Heart Infusion (Difco, Detroit, MI) supplemented with 1 µg/ml of 1% thiamine monophosphate (Sigma, St. Louis, MO), and 10 µg/ml of 10% Trizma base (Sigma, St. Louis, MO)], and incubated aerobically for 10 h at 37C on a shaker. As the final step, this broth culture was diluted to 1:4 with BHITT and incubated for 2 h until the optical density (OD) (660 nm) reached 0.4. One ml of this broth was found to contain approximately $1X10^9$ logarithmically growing *H. somnus*. The actual number of bacteria per ml was determined by plating 10-fold serial dilutions of the suspension in duplicate and counting colonies. This number was used to calculate the ratio of blood leucocytes to bacteria in all experiments. *H. somnus* was recovered and washed twice

with cold (4C) Hanks balanced salt solution (HBSS) by centrifugation before being used. Logarithmically growing *H. somnus* was killed by heating at 65C for 30 min.

Source of Bovine Blood

Heparinized (Organon Teknica, Toronto, Canada) bovine blood was collected into 60 ml plastic syringes (Becton Dickinson, Rutherford, NJ) from clinically normal, 6-12 month-old beef calves. These animals were maintained at V.I.D.O. research facilities at the University of Saskatchewan.

The H. somnus Bovine Experimental Model

A field isolate of *H. somnus* (HS25) was injected intravenously into 6-8 month-old beef calves. (Harland *et al.*, 1990; Schuh *et al.*, 1991). The challenge dose contained approximately $5X10^7$ colony forming units (CFU). Clinical signs occurring after challenge included fever, depression, and polyarthritis. All the animals were necropsied within a 14 day period. The challenge was fatal to 60%-70% of the calves and the necropsy findings included meningitis, encephalitis (Gomis *et al.*, 1993), myocarditis, pericarditis, pneumonia, pleuritis and polyarthritis. This bovine *H. somnus* model closely reproduced the clinical and pathological features of the disease as seen in Western Canada.

For TNF, IFN-γ, and IL-6 determination, blood samples were taken from 8 experimentally infected animals on each of days -5, -4, 0, 1, 2, 3, 4, and 7 post-infection.

Blood samples collected into vacutainer tubes (Becton Dickinson Vacutainer

Systems, Rutherford, NJ) from 8 experimentally infected cattle with *H. somnus* were centrifuged and the separated serum which was stored at -70C until the time of cytokine assays. Additionally, a sample of heparinized blood was obtained from same experimentally infected cattle with *H. somnus* was infected by *in vitro* addition of *H. somnus* and plasma samples were separated and stored at -70C until the time of cytokine assays.

Whole-Blood Culture

The heparinized whole-blood cultures were set up in a volume of 3 ml in polypropylene tubes (4.5 ml, Becton Dickinson, Rutherford, NJ), as previously described (Yachie *et al.*, 1992). The cultures were maintained at 37C in an atmosphere containing 5% CO_2 on a nutator (Clay Adams, Becton Dickinson Co, NJ). At the beginning of the culture, live or killed *H. somnus* were added directly to the heparinized whole blood at the ratio of 1:10 (white blood cell:*H. somnus*). After 0, 8, and 24 h incubation with live or heat killed *H. somnus*, plasma was separated from the cultured blood by centrifugation at 900 g at 4C for 10 min. The plasma was diluted 1:4 with minimum essential medium (MEM) (Gibco BRL, Life Technologies Inc. Grand Island, NY) supplemented with 1% fetal bovine serum (heat inactivated) (Gibco BRL, Life Technologies Inc. Grand Island, NY), and stored at -70C until the time of cytokine assays.

IL-6 Bioassay

The IL-6 content of tested samples was assayed by monitoring the ability of the

samples to cause proliferation of the IL-6-dependent mouse hybridoma cell line 7TD1 (American Type Culture Collection, Rockville, MD). Briefly, 7TD1 cells were propagated in MEM supplemented with 2 ng/ml of recombinant human IL-6 rHuIL-6 (Genzyme Diagnostics, Cambridge, MA) in 25 cm^2 flasks (Corning, New York, NY). This assay was conducted in flat bottom 96 well microtitre plates (Costar, Cambridge, MA). Five thousand 7TD1 cells were cultured in microtitre wells in 100 µl of G-MEM [MEM supplemented with 2 nM L-glutamine (Gibco BRL, Life Technologies Inc. Grand Island, NY), 50 mM 2-mercaptoethanol (Sigma Chemical Co. St. Louis, MO), 25 mM Hepes (Gibco BRL, Life Technologies Inc. Grand Island, NY), 1 mM Sodium pyruate (Gibco BRL, Life Technologies Inc. Grand Island, NY), 1% non essential amino acids (Gibco BRL, Life Technologies Inc. Grand Island, NY), and 10% heat inactivated fetal bovine serum (Gibco BRL, Life Technologies Inc. Grand Island, NY)]. Then 100 µl volumes of test culture supernatants were added to the wells in duplicate, and the plates were incubated for 96 h 37C in an atmosphere containing 5% CO_2. Dilutions of rHuIL-6 were included as the standard. After 96 h incubation, microtitre plates were centrifuged for 5 min at 900 g, 100 µl of medium was removed, and 25 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml in phosphate buffered saline) (Sigma Chemical Co. St. Louis, MO) was added and plates were incubated for 2 h at 37C in an atmosphere containing 5% CO_2 to allow viable 7TD1 cells to reduce MTT to formazan. Then 100 µl of extraction buffer containing SDS (sodium dodecyl sulphate) (Sigma Chemical Co. St. Louis, MO), and N,N-dimethylformamide (Sigma Chemical Co. St. Louis, MO) (Hansen *et al.*, 1989) were added to the microtitre wells and plates were incubated for 2 h at 37C in an

atmosphere containing 5% CO_2 to dissolve the purple formazan precipitate. Formazan produced by viable 7TD1 cells was quantified by measuring OD with wavelength of 595 nm (reference 655 nm) using an automated microtitre plate reader (Bio-Rad Laboratories, model 3550, Richmond, CA).

TNF Bioassay

The activity of TNF-α in samples was determined by measuring their cytotoxicity for murine connective tissue origin, fibroblast-like L929 (American Type Culture Collection, Rockville, MD) cells as described previously by Havell (1989). This assay was conducted in flat bottom 96 well microtitre plates (Costar, Cambridge, MA). Briefly, $5X10^4$ of L929 cells in 100 µl of MEM were added to each microtitre well and incubated for 24 h at 37C in an atmosphere containing 5% CO_2. Then the media was removed from all wells and replaced with 100 µl of MEM containing 10% fetal bovine serum, and 2 µg/ml Actinomycin D (Sigma Chemical Co. St. Louis, MO). As the next step, 100 µl of samples or standards were added to microtitre wells in quadruplicate and incubated for 24 h at 37C in an atmosphere containing 5% CO_2. Recombinant bovine TNF-α (Genentech Inc., San Francisco, CA) was used as a standard control. After 24 h incubation, 100 µl of medium was removed and 25 µl of MTT (5 mg/ml in phosphate buffered saline) was added and the plates were incubated for 2 h at 37C in an atmosphere containing 5% CO_2 to allow viable L929 cells to reduce MTT to formazan. Then 100 µl of extraction buffer containing SDS, and N,N-dimethylformamide (Hansen *et al.*, 1989) were added to the microtitre wells and the plates incubated for 2 h at 37C in an atmosphere containing 5% CO_2 to dissolve purple

formazan precipitate. Formazan produced by viable L929 cells were quantitated by measuring OD with wavelength of 595 nm using an automated microtitre plate reader (Bio-Rad Laboratories, model 3550, Richmond, CA).

TNF Capture ELISA

The methodology used in this assay was previously described by Ellis *et al.* (1993). Briefly one hundred µl of mouse anti-TNF ascitic fluid (1D11-13) diluted 1/1000 in carbonate coating buffer (0.05 M sodium carbonate, pH 9.6) (BDH Inc. Toronto, ON) were adsorbed to Immunolon-II plates (Dynatech Laboratories, Inc., Chantilly, VA) at 4C for 12 h. Then the plates were washed four times with distilled water. One hundred µl of serial dilutions of serum samples or rBoTNF in FBS (Fetal bovine serum) (standard) (Genentech Inc., San Francisco, CA) were added to each well and incubated for 2 h in room temperature. After 2 h incubation, plates were washed four times with distilled water and 100 µl/well of rabbit anti-TNF IgG diluted 1/1000 added as the detection antibody and plates were incubated 1 h at room temperature. Plates were then washed four times in distilled water and goat anti-rabbit Alkaline Phosphate Conjugate (Kirkegaard and Perry Laboratories Inc., Gaithersbury, MD) diluted 1/5,000 in diluent was added (100 µl/well) and incubated for 1 h at room temperature. After four washes in distilled water, PNPP substrate (1 mg PNPP per ml of 1% Diethanolamine with 0.5 mM $MgCl_2$, pH 9.8) (Sigma Chemical Co. St. Louis, MO) (100 µl/well) was added and plates were incubated at room temperature for 2 h. After 2 h prior to stopping the reaction with 30 µl of 0.3M EDTA (Ph 8.0) (Sigma Chemical Co. St. Louis, MO) and measuring OD at 405 (reference 495 nm) in a

BioRad 3550 Microplate reader (BioRad, Richmond, CA).

IFN-γ Capture ELISA

One hundred µl of mouse anti-rBoIFN-γ mAb (recombinant bovine IFN-γ monoclonal antibodies) diluted 1/4000 in carbonate coating buffer (0.05 M sodium carbonate, pH 9.6) (BDH Inc. Toronto, ON) were adsorbed to Immunolon-II plates (Dynatech Laboratories, Inc., Chantilly, VA) at 4C for 12 h. Then the plates were washed six times with PBST (Phosphate buffered saline supplemented with 0.05% Tween-20) (Fisher Scientific Co., Fairlawn, NJ) and samples (1:2 dilutions in PBST) or standards in fetal bovine serum (heat inactivated) were added. Two hundred µl of serial dilutions of rBoINF-γ (from 2 ng/ml to - 10 ng/ml) (Ciba-Geigy, Basle, Switzerland) were added to each well as the standard. Fetal bovine serum and empty wells were used as negative controls and blanks, respectively. Then plates were incubated at room temperature for 2 h. After 2 h incubation, plates were washed six times with PBST and 100 µl/well of rabbit anti-IFN-γ diluted 1/1000 in PBST-g (PBS supplemented with 0.5% gelatin) was added as the detection antibody and plates were incubated 1 h at room temperature. Plates were then washed six times in PBST and biotinylated goat anti-rabbit IgG (Zymed, San Francisco, CA) diluted 1/10,000 in PBST-g was added (100 µl/well) and incubated for 1 h at room temperature. After six washes in PBST, horseradish peroxidase-conjugated streptavidin-biotin complex (Amersham, Arlington Height IL) diluted 1/2000 in PBST-g (100 µl/well) was added and plates were incubated at room temperature for 1 h. Following six washes with PBST, 100 µl of ABTS substrate (2,2'-Azino-di[ethyl-benzthiazolinsulfonate (6)]

(Boehringer, Mannheim, Germany) was added and plates were incubated for 30 min at room temperature prior to stopping the reaction with 50 µl of 10% SDS (Sigma Chemical Co. St. Louis, MO) and measuring OD at 405 (reference 490 nm) in a BioRad 3550 Microplate reader (BioRad, Richmond, CA).

RESULTS

Ex vivo production of TNF, IFN-γ, and IL-6 with the interaction of *H. somnus*

No TNF was detected regardless of whether or not *H. somnus* was present. When blood cultures were stimulated with live *H. somnus*, IFN-γ (Fig. 1) and IL-6 (Fig. 2) became detectable at 8 h and were maintained at that level for up to 24 h. With the addition of heat killed *H. somnus*, no IFN-γ or IL-6 was detectable until 24 h.

Production of TNF, IFN-γ, and IL-6 in Blood from Cattle Experimentally Infected with *H. somnus*

Production of TNF, IFN-γ or IL-6 was not found in serum of cattle experimentally infected with *H. somnus* between 0 and 7 days post-infection. With *in vitro* addition of *H. somnus* into whole blood cultures prepared from cattle experimentally infected with *H. somnus*, TNF production was not detectable during the 24 h incubation period, but IFN-γ reached a was detectable (Fig. 3). The *ex vivo* IFN-γ response to *H. somnus* in whole blood cultures was temporally inhibited in calves at day 2 post-infection, the time when most of the death resulting (4 out of 8 calves died)

from infection occurred, and recovered from day 3 with the maximal response seen on day 7 post-infection. The course of IL-6 production (Fig. 4) followed a slightly different pattern from that of IFN-γ, in that the IL-6 response was inhibited in the calves on days 1 and 2 post-infection, maximal on day 3 and then waned but maintained at a substantial level throughout the experiment.

DISCUSSION

The objective of this study was to detect the endogenous TNF, IFN-γ, and IL-6 response to *H. somnus* infection; (1) using serum samples of cattle experimentally infected with *H. somnus* (*in vivo*) and (2) plasma samples following *in vitro* exposure to *H. somnus* (*ex vivo*). Factors that might contribute to the differences of cytokine profiles observed between *in vivo* and *ex vivo* results may include: presence or absence of a bacteremic state in experimentally infected animals; differences in the length of time that leucocytes were exposed to *H. somnus;* the ratio of bacteria to leucocytes within the animal; the potential availability of cytokine inhibitors in the circulation; differences in magnitude of inflammatory mediators; or localization of these cytokines in lesions in contrast to elevated levels in circulation.

The bacteremia of experimentally infected cattle with *H. somnus* follows a multi-phasic pattern with unpredictable intervals between phases (Harland, R. personal communication). Due to the unpredictable nature of bacteremia in this bovine animal model, *H. somnus* might not have been available to interact with leucocytes to induce cytokines in the blood. This possibility is supported by the observation that IFN-γ and IL-6 were produced after *in vitro* addition of *H. somnus* to the blood obtained from

cattle experimentally infected with *H. somnus*. Thus, this rules out the possibility that a dormant state was induced in leucocytes due to the interaction with *H. somnus*. Undetectable TNF induction by *H. somnus*, both *in vivo* and *ex vivo* might be associated with intracellular survival of *H. somnus*, since TNF is known to be a activator of microbial killing mechanisms of macrophages. It is still a possibility that transient induction of TNF in the early infection of *H. somnus* might have not been detected in these experiments. Since both the bioassay and the capture ELISA were unable to detect TNF in these experiments, it is unlikely that significant levels of TNF were produced. It has been reported the ability of rBo-IFN-γ to clear *H. somnus* infection in experimentally infected cattle (Chiang *et al.*, 1990). Production of IFN-γ might have been associated with the clearance of the *H. somnus* infection; this possibility is supported by the correlation between temporary inhibition of IFN-γ production in calves at day 2 post-infection where highest mortality occurred.

The importance of these cytokine profiles in relation to clearance of the infection is not known. Since all eight of the experimental animals either died or were euthanized for humane reasons between day 1 and 10 post-infection, it was not possible to examine the cytokine profiles in recovered animals. Further, no recombinant bovine cytokines were injected into these animals to evaluate potential recovery or resistance to infection. However, during *in vitro* studies using rBoIFN-γ or rBoTNF-α to activate bovine blood monocytes and alveolar macrophages, these cells were not able to kill intracellular *H. somnus* (chapter 4). Identification of cytokine profiles in *H. somnus* hyperimmune or resistant animals following *H. somnus* inoculation might be beneficial in understanding the immunological mechanisms important for disease

resistance.

Fig. 1: Production of IFN-γ following *in vitro* addition of live *H. somnus* (L-som) and killed *H. somnus* (K-som) into whole blood cultures from clinically normal animals. *H. somnus* was added into whole blood cultures at the beginning of the assay and plasma samples were collected at 0, 8, and 24 h. later for IFN-γ detection. No IFN-γ was detected in control cultures. Each value shown is mean and standard deviation. (n=5)

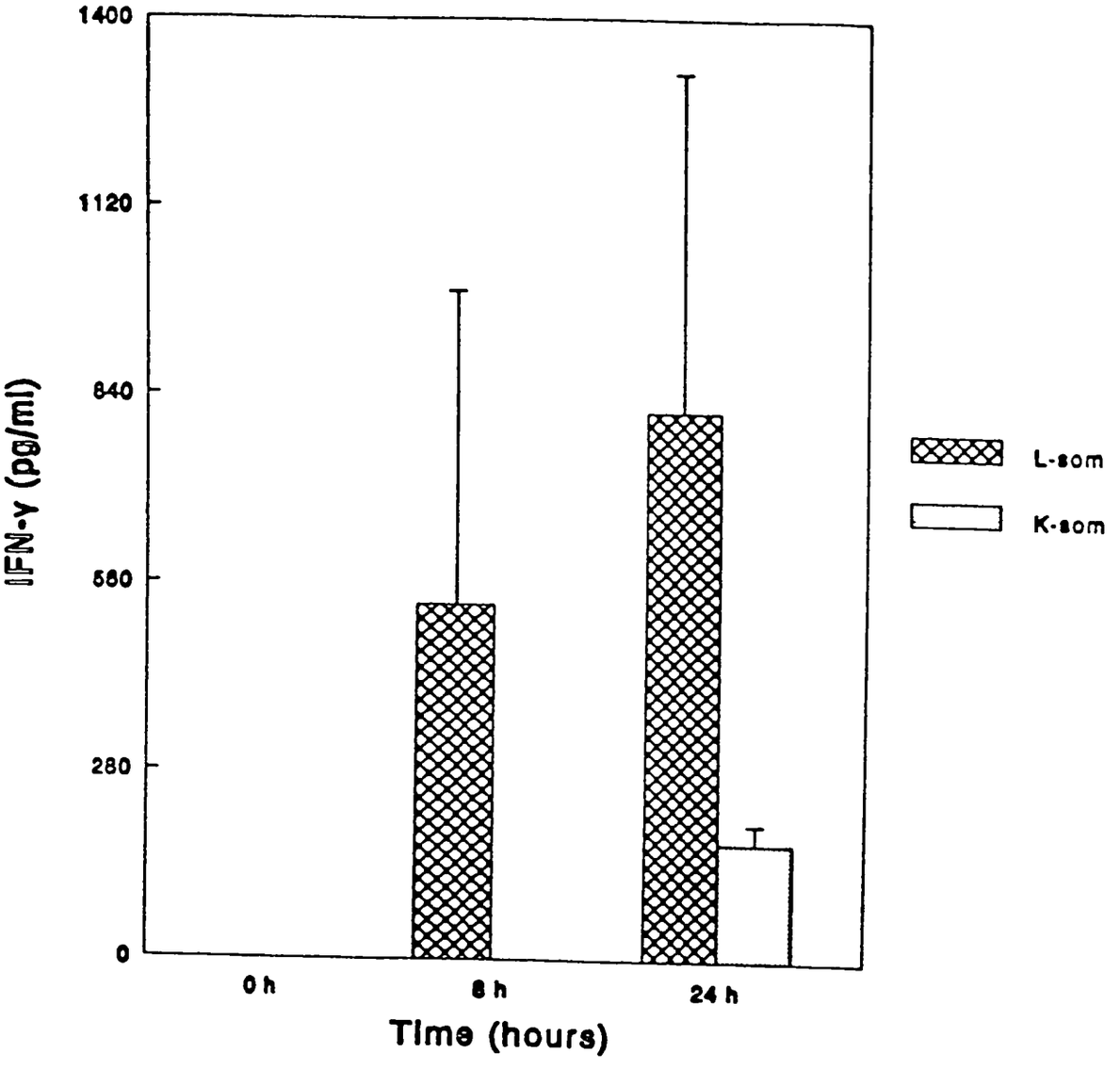

1400
1120
840
560
280
0
IFN-γ (pg/ml)
0 h
8 h
24 h
Time (hours)
L-som
K-som

Fig. 2: Production of IL-6 following *in vitro* addition of live *H. somnus* (L-som) and killed *H. somnus* (K-som) into whole blood cultures from clinically normal animals. *H. somnus* was added into whole blood cultures at the beginning of the assay and plasma samples were collected at 0, 8, and 24 h. later for IL-6 detection. No IL-6 was detected in control cultures. (n=5) Each value shown is mean and standard deviation.

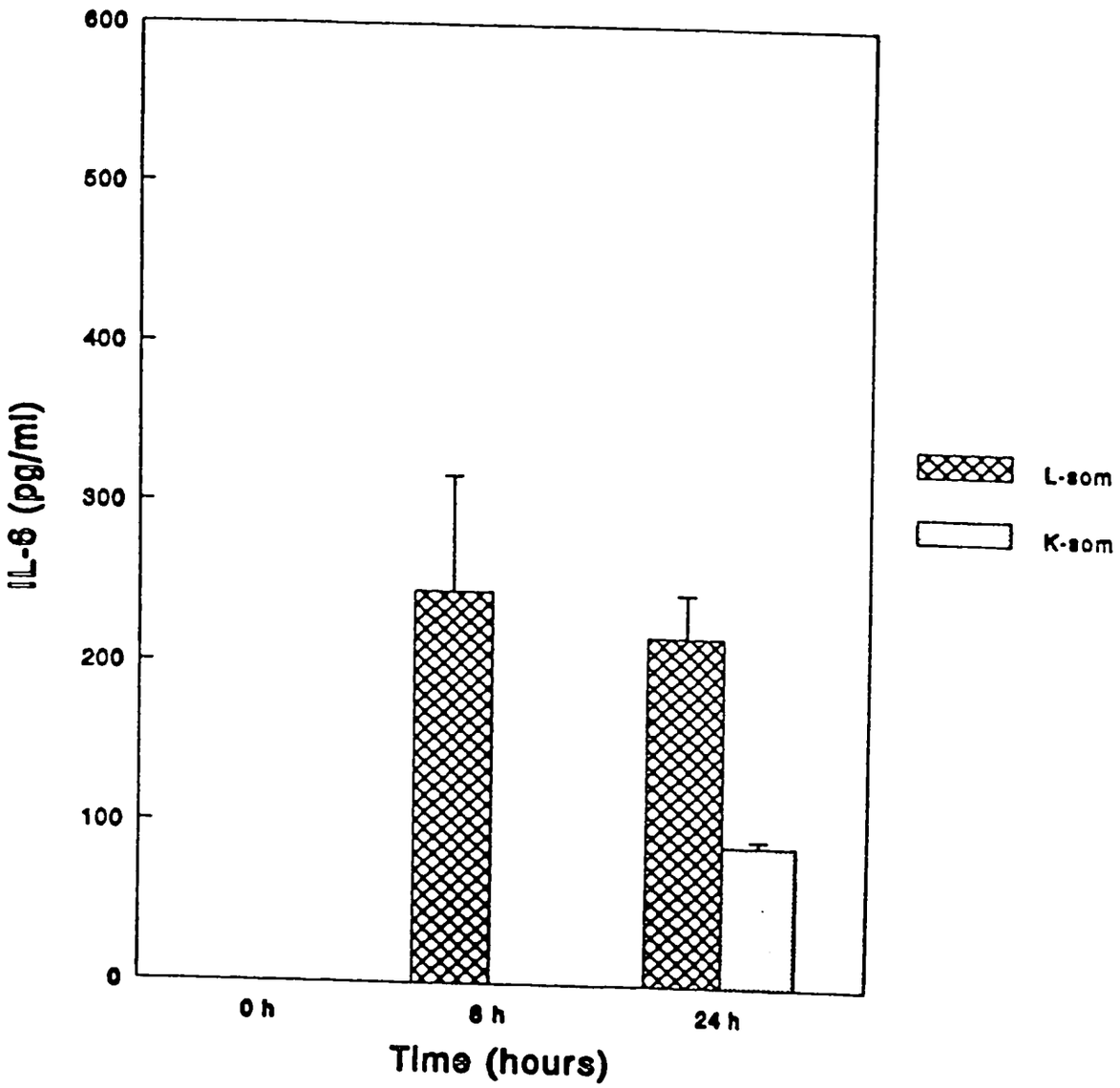

600
500
400
300
200
100
0
IL-6 (pg/ml)
0 h
8 h
24 h
Time (hours)
L-som
K-som

Fig. 3: Production of IL-6 following *in vitro* addition of *H. somnus* into whole blood cultures obtained from cattle experimentally infected with *H. somnus*. *H. somnus* was added into whole blood cultures at the beginning of the assay and plasma samples were collected at 8, and 24 h. later for IL-6 detection. Note the IL-6 response on day 2 post-infection where 4 out of 8 animals either died or euthanized. [0, 8, and 24 h = detection of IL-6 at 0, 8, and 24 h after *in vitro* addition of live *H. somnus* into whole blood cultures] Each value shown is mean and standard deviation. (n=8)

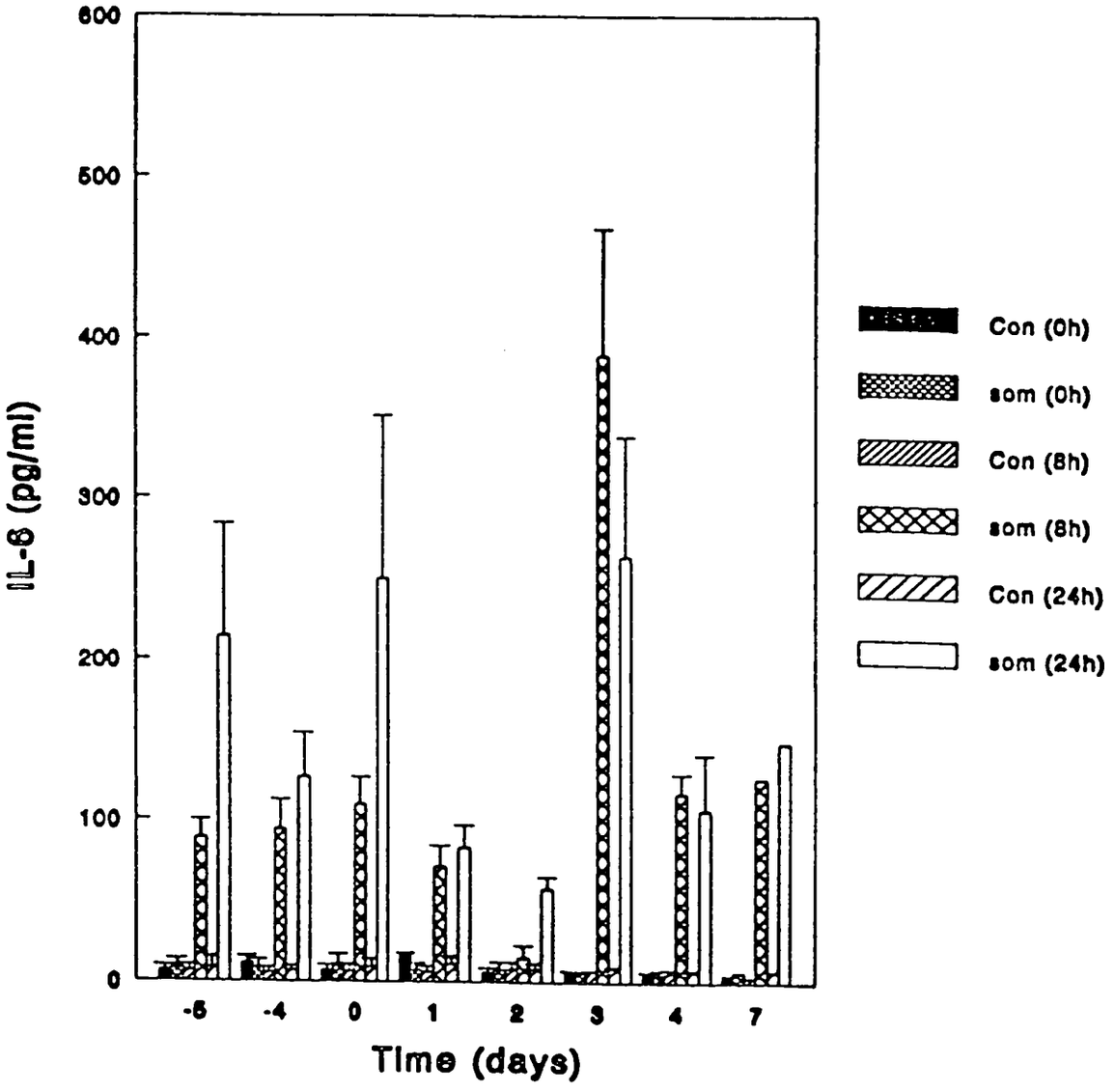
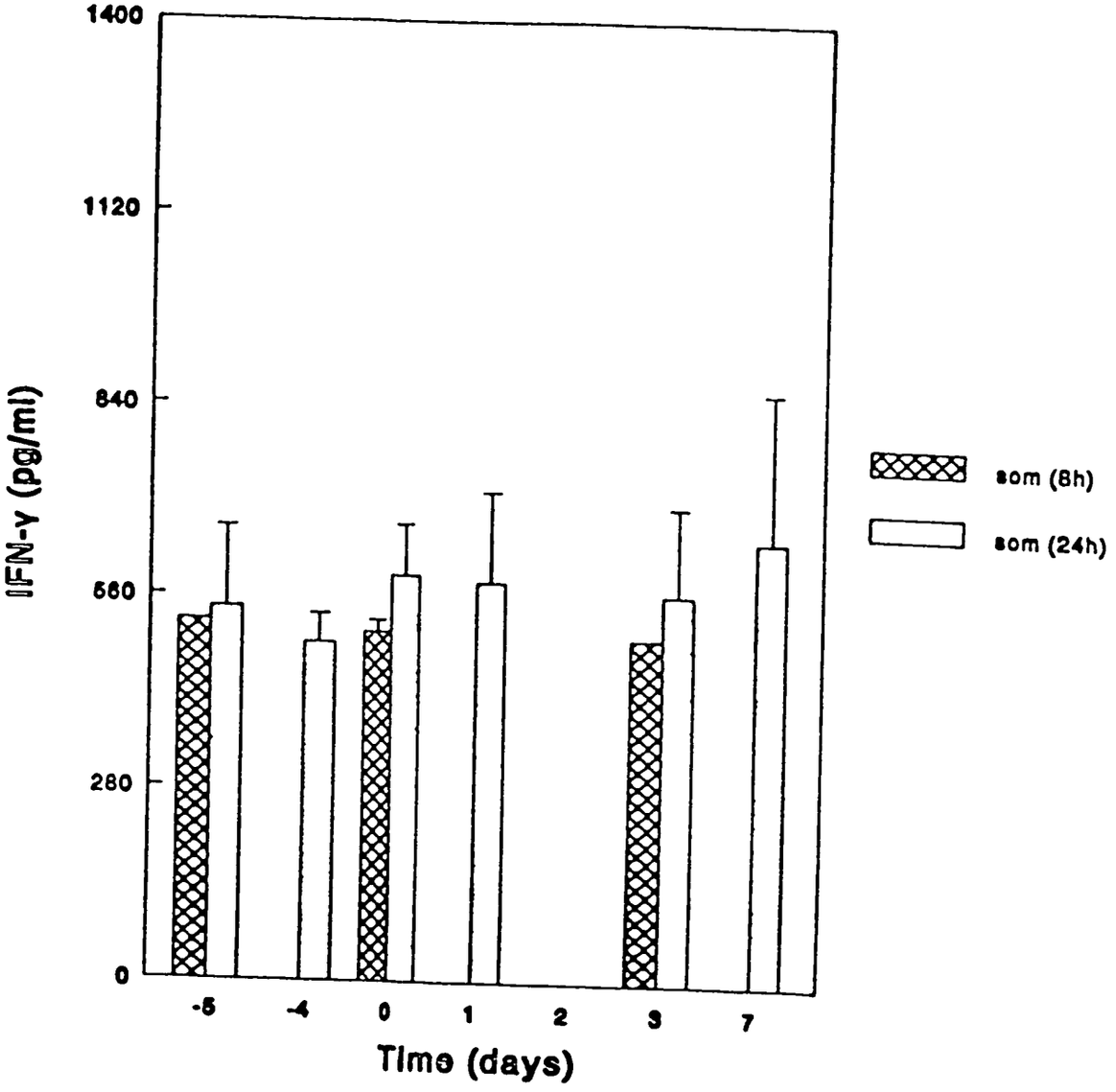

600
500
400
300
200
100
0
IL-6 (pg/ml)
-5
-4
0
1
2
3
4
7
Time (days)
Con (0h)
som (0h)
Con (8h)
som (8h)
Con (24h)
som (24h)

Fig. 4: Production of IFN-γ following *in vitro* addition of *H. somnus* into whole blood cultures obtained from cattle experimentally infected with *H. somnus*. *H. somnus* was added into whole blood cultures at the beginning of the assay and plasma samples were collected at 8, and 24 h later for IFN-γ detection. Note the IFN-γ response on day 2 post-infection where 4 out of 8 animals either died or euthanized. No IFN-γ was detected in control cultures at any of the sampling period. No IFN-γ was detected at 0 h after *in vitro* addition of *H. somnus* into whole blood cultures. [0, 8, and 24 h = detection of IFN-γ at 0, 8, and 24 h after *in vitro* addition of live *H. somnus* into whole blood cultures] Each value shown is mean and standard deviation. (n=8)


1400
1120
840
560
280
0
IFN-γ (pg/ml)
-5
-4
0
1
2
3
7
Time (days)
som (8h)
som (24h)

7. SUMMARY AND CONCLUSIONS

The objective of this study was to investigate the interaction between BMP and *H. somnus*. Broadly, four approaches were undertaken for the investigation of these interactions. The first was to investigate the effect of *H. somnus* on phagocytosis by BMP, both *in vitro* and *ex vivo*. The second approach was to investigate the bacterial killing mechanisms of BMP following interaction with *H. somnus*. Recombinant bovine cytokines including rBoIFN-γ, rBoTNF-α, rBoGM-CSF, and rBoIL-1β were used to stimulate the macrophages in these *in vitro* studies. Ultrastructural studies and bactericidal assays were conducted to investigate the intracellular survival kinetics of *H. somnus* in BMP. The third approach was directed towards demonstrating bactericidal ability of BMP following interaction with *H. somnus*. The fourth approach was directed towards demonstrating the cytokine profile of BMP following interaction with *H. somnus*.

To study the effect of *H. somnus* on phagocytosis by BMP, a flow cytometric assay was developed. Using this *in vitro* system, it was found that logarithmically growing *H. somnus* significantly inhibited the phagocytosis of opsonized *S. aureus* by BAM obtained both from healthy calves and from cattle experimentally infected with *H. somnus*. However, neither heat- or formalin-killed, log-phase *H. somnus*, nor *in vitro* passaged *H. somnus* had any effect on the phagocytic activity of these cells. In contrast to BAM, BBM had a significant increase in their phagocytic activity following *in vitro* exposure to logarithmically growing *H.somnus*. These results indicate that *H.*

somnus has the ability to modulate the phagocytic function of both BBM and BAM. The ability of *H. somnus* to modulate BAM/BBM phagocytic function may contribute towards the pathogenesis of the disease.

The second approach was to study the mechanisms used by *H. somnus* to survive and multiply within BMP. To study the interaction between BMP and *H. somnus*, a colorimetric assay using MTT was developed to assess the survival of *H. somnus* within cultured BBM. Using this system, it was found that: (1) *H somnus* was able to survive within BBM *in vitro*, and the kinetics of its survival were similar to that seen in BBM isolated from experimentally infected cattle; (2) treatment of BBM with rBoIFN-γ, rBoTNF-α, rBoIL-1β, rBoGM-CSF and *E. coli* LPS had no significant effect on the survival of *H. somnus*. Moreover, using ultrastructural studies, and ^{3}H uracil incorporation into nucleic acids, it was also possible to confirm the survival of *H. somnus* in BMP. The ability of *H. somnus* to survive and multiply in both freshly isolated and cytokine treated BBM could be an explanation for chronic haemophilosis since BMP could provide a shelter for this intracellular pathogen.

The third approach was to study the bactericidal ability of BMP following interaction with *H. somnus*. Two *in vitro* assay systems were developed to detect NO production and LDCL of BBM and BAM. *H. somnus* rapidly inhibited the LDCL of BBM and BAM costimulated with opsonized *Staphylococcus aureus*. The kinetics of the inhibitory effect of *H. somnus* on BBM and BAM LDCL were similar. Inhibition of the LDCL response of BBM and BAM was not seen with either opsonized or killed *H. somnus*. The ability of *H. somnus* to inhibit LDCL of both BBM and BAM may be an important virulence mechanism that could contribute to the survival of the organism

following ingestion by BMP. To study the kinetics of NO production by BMP, various bovine recombinant cytokines and *E. coli* LPS were used. Recombinant BoIL-1β and *E. coli* LPS were better inducers of NO production by BBM and BAM than was rBoGM-CSF. Both BBM and BAM were stimulated to produce NO after exposure to *H. somnus*. Since *H. somnus* was able to survive intracellularly within BMP while inducing NO, it is not known what advantage induction of NO production might have for intracellular survival of *H. somnus*.

To determine the cytokines produced following the interaction of *H. somnus* with leukocytes, a whole blood culture assay system was developed. Blood samples from healthy calves were incubated with or without the presence of *H. somnus* for up to 24 h. No tumor necrosis factor (TNF) was detected regardless of whether or not live *H. somnus* was present. When stimulated with live *H. somnus*, IFN-γ and interleukin-6 IL-6 became detectable at 8 h and were maintained at a detectable level for up to 24 h whereas, with the simulation of heat killed *H. somnus* no detectable level of IFN-γ or IL-6 was found at 8 h but these cytokines became detectable at 24 h. No detectable TNF, IFN-γ or IL-6 was found in serum of cattle experimentally infected with *H. somnus* between 0 and 7 days post-infection. With *in vitro* addition of *H. somnus* into whole blood cultures prepared from cattle experimentally infected with *H. somnus*, TNF production was not detectable during the 24 h incubation period. In contrast, IFN-γ reached a detectable level. In addition, the *ex vivo* IFN-γ response to *H. somnus* in whole blood cultures was inhibited in calves killed at day 2 post-infection, a time when most deaths occurred as a result of the infection, and recovered from day 3 on with the maximal response occurring on day 7 post-infection. The course of IL-6 production

followed a slightly different pattern from that of IFN-γ, in that the IL-6 response was inhibited in calves on days 1 and 2 post-infection, maximal on day 3 and then waned but maintained at a substantial level throughout the remainder of the experiment. The temporary inhibition of IFN-γ and IL-6 on day 2 post-infection might be associated with the poor ability of clearing the infection. Intracellular survival and multiplication of *H. somnus* in BMP could be responsible for these cytokine profiles.

This study reconfirmed *H. somnus* as an intracellular pathogen of BMP using ultrastructural studies, a colorimetric bactericidal assay and a ^{3}H uracil assay. Ultrastructural studies demonstrated the membrane bound *H. somnus* in the cytoplasm of both BBM and BAM. The phagosome-lysosome fusion was not studied to further characterize the intracellular survival mechanism. The ability of unopsonized log and stationary phase *H. somnus* to enter BMP might be important in dissemination of the organism in the body and evasion from humoral immune system. Inhibition of the LDCL response of both BBM and BAM by either logarithmically growing or stationary phase *H. somnus* might be crucial for intracellular survival. Moreover, live *H. somnus* alone induce a very low LDCL response compared to killed *H. somnus*. This difference might be associated with scavenging of reactive oxygen metabolites by *H. somnus*, although *H. somnus* is a catalase negative organism. The mechanisms involved with reduction of LDCL response of BMP were not studied.

This study explains the interaction of *H. somnus* with BMP to a certain extent. Since an important control method for this disease syndrome is vaccination, it is important to recognize the importance of both humoral and cell mediated immunity for protection. Antibodies which are able to opsonize the pathogen may not be sufficient

to prevent disease in this case, since it is an intracellular pathogen. The antibodies that can interact with antigens of *H. somnus* which are important in intracellular survival might be important for protection. Further studies are necessary to investigate the virulence factors of *H. somnus* which are responsible for this intracellular survival.

REFERENCES

Abou-Zeid, C., Ratliff, T.L., Wiker, H.G., Harboe, M., Bennedsen, J., Rook, G.A. (1988) Characterization of fibronectin-binding antigens released by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG. Infect. Immun. **56(12)**: 3046-3051.

Adams, J., and Czuprynski, J. (1990) Bacterial lipopolysaccharide induces release of tumor necrosis factor-α from bovine peripheral blood monocytes and alveolar macrophages *in vitro*. J. Leukoc. Biol. **48**: 549-556.

Adams, L.B., Hibbs, J.B.Jr., Taintor, R.R., and Krahenbuhl, J.L. (1990) Microbiostatic effect of murine activated macrophages for *Toxoplsma gondii*. Role for synthesis of inorganic nitrogen oxides from L-arginine. J. Immunol. **144**: 2725-2729.

Aderem, W., Wright, S.D., Silverstein, S.C., Cohn, Z.A. (1985) Ligated complement receptors do not activate the arachidonic acid cascade in resident peritoneal macrophages. J. Exp. Med. **161**: 617-622.

Alexander, J., and Vickerman, K. (1975) Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of *Leishmania mexicana* infected macrophages. J. Protozool. **22**: 502-508.

Allan, R.C. (1977) Evaluation of serum opsonic capacity by quantitating the initial chemiluminescent response from phagocytizing polymorphonuclear leucocytes. Infect Immun. **15**: 828-833.

Allen, R.C. (1979) Reduced, radical, and exited state oxygen in leucocyte microbicidal activity. Front. Biol. **48**: 197-233.

Allen, R.C., Loose, L.D. (1976) Phagocytic activation of a luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. Biochem. Biophys. Res. Commun. **69**: 245-252.

Allison, A.C., (1978) Mechanisms by which activated macrophages inhibit lymphocyte responses. Immunol. Rev. **40**: 3-27.

Ames, T.R. (1987) Neurologic disease caused by *Haemophilus somnus*. Vet. Clin. North. Am. [Food Anim Pract] **3**: 61-73.

Andrews, J.J., Anderson, T.D., Slife, L.N., and Stevenson, G.W. (1985) Microscopic lesions associated with the isolation of *Haemophilus somnus* from pneumonic

bovine lungs. Vet. Pathol. **22**: 131-136.

Andrews, J.J., Slife, L.N., and Stevenson, G.W. (1983) Naturally occurring *Haemophilus somnus* in cattle. Proc. III Int. Vet. Lab. Diagnost. 377-384.

Andus, T., Geiger, T., Hirano, T., Kishimoto, T., Tran-Thi, T.-A., Decker, K., and Heinrich, P.C. (1988) Regulation of synthesis and secretion of major rat acute-phase proteins by recombinant human interleukin 6 (BSF-2/IL-6) in hepatocyte primary cultures. Eur. J. Biochem. **173**: 287-293.

Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N., and Yokota, T. (1990) Cytokines: Coordinators of immune and inflammatory responses. Annu. Rev. Biochem. **59**: 783-836.

Armstrong, J. A., and Hart, P.D. (1971) Response of cultured macrophages to *Mycobacterium tuberculosis* with observations on fusion of lysosomes with phagosomes. J. Exp. Med. **134**: 713-740.

Armstrong, K.R., Osborne, A.D., and Janzen, E.D. (1986) *Haemophilus somnus* mastitis in a dairy cow. Can. Vet. J. **27**: 211-212.

Babior, B.M. (1978). Oxygen-dependant microbial killing by phagocytes. N. Engl. J. Med. **298**: 659-668.

Badwey, J.A., and Karnovesky, K.L. (1980) Active oxygen species and the function of phagocytic leucocytes. Ann. Rev. Biochem. **49**: 695-726.

Bailie, W.E (1969) Characterization of *Haemophilus somnus* (new species), a microorganism isolated from infectious thromboembolic miningoencephalitis in cattle. Diss. Abst. **30(B)**, 2482.

Bailie, W.E., Anthony, H.D., and Weide, K.D. (1966) Infectious embolic meningoencephalitis (sleeper syndrome) in feedlot cattle. J. Am. Vet. Med. Assoc. **148**: 162-166.

Bailie, W.E., Coles, E.H., and Weide, K.D. (1973) Deoxyribonucleic acid characterization of a microorganism isolated from infectious thromboembolic meningoencephalitis in feedlot cattle. Int. J. Syst. Bacteriol. **23(3)**: 231-237.

Baldwin, C.L., Jiang, X. and Fernandes, D.M. (1993) Macrophage control of *Brucella abortus*: influence of cytokines and iron. Trends. Microbiol. **1(3)**: 99-104.

Bancroft, G.J., Schreiber, R.D., and Unanue, E.R. (1991) Natural immunity: a T-cell-dependent pathway of macrophage activation defined in the scid mouse. Immunol. Rev. **124**: 5-24.

Barnes, P.F., Fong, S.J., Brennan, P.J., Twomey, P.E., Mazumder, A., and Modlin, R.L. (1990) Local production of tumor necrosis factor and interferon-γ in tuberculous pleuritis. J. Immunol. **145**: 149-154.

Barnum, D.A. (1990) Socioeconomic significance of the HAP group. Can. J. Vet. Res. **54(supplement)**: s1-s5.

Beaman, B.L., Black, C.M., Doughty, F., and Beaman, L. (1985) Role of superoxide dismutase and catalase as determinants of pathogenicity in *Nocardia asteroides*: importance in resistance to microbicidal activities of human polymorphonuclear neutrophils. Infect. Immun. **47**: 135-141.

Berche, P., Gaillard, J.L., and Sansonetti, P.J. (1987) Intracellular growth of *Listeria monocytogenes* as a prerequisite for *in vivo* induction of T cell-mediated immunity. J. Immunol. **138**: 2266-2271.

Berman, J.D., Dwyer, D.M., Wyler, D.J., (1979) Multiplication of *Leishmania* in human macrophages *in vitro*. Infect. Immun. **26**: 375-379.

Bermudez, L.E., Petrofsky, M., Wu, M., and Young, L.S. (1992) Interleukin-6 antagonizes Tumor necrosis factor-mediated Mycobacteriostatic and Mycobactericidal activities in macrophages. Infect. Immun. **60**: 4245-4252.

Bermudez, L.E., Stevens, P., Kolonoski, P., Wu, M., and Young, L.S. (1989) Treatment of disseminated *Mycobacterium avium* complex infection in mice with recombinant interleukin-2 and tumor necrosis factor. J. Immunol. **143**: 2996-3002.

Bermudez, L.E.M., and Young, L.S. (1988) Tumor necrosis factor, alone or in combination with IL-2, but not IFN-γ, is associated with macrophage killing of *Mycobacterium avium* complex. J. Immunol. **140**: 3006-3013.

Bermudez, L.E., and Young, L.S. (1989) Oxidative and non-oxidative intracellular killing of *Mycobacterium avium* complex. Microb. Pathog. **7**: 289-297.

Bermudez, L.E.M., and Young, L.S. (1990) Recombinant granulocyte-macrophage colony-stimulating factor activates human macrophages to inhibit growth or kill *Mycobacterium avium* complex. J. Leucocyte. Biol. **48**: 67-73.

Bermudez, L.E., Young, L.S., and Enkel, H. (1991) Interaction of *Mycobacterium avium* complex with human macrophages: roles of membrane receptors and serum proteins. Infect. Immun. **59**: 1697-1702.

Berton, G., Dusi, S., and Bellavite, P. (1988) The respiratory burst of phagocytes. In the respiratory burst and it's physiological significance. A.J. Sbarra and R.R.

Strauss, ed. Plenum Press, New York, p33.

Betz, M., and Fox, B.S. (1991) Prostaglandin E_2 inhibits production of Th-1 lymphokines but not Th-2 lymphokines. J. Immunol. **146:** 108-113.

Beutler, B. (1990) TNF in pathophysiology-biosynthetic regulation. J. Invest. Dermatol. **95:** s81-s84.

Beutler, B., Milsark, I.W., and Cerami, A. (1985) Passive immunization against cachectin/tumor necrosis factor (TNF) protects mice from the lethal effect of endotoxin. Science **229:** 869-871.

Bevilacqua, M.P., Amrani, D., Mosesson, M.W., Bianco, C. (1981) Receptor for cold insoluble globulin (Plasma fibronectin) on human monocytes. J. Exp. Med. **153:** 42-60.

Bhardwaj, N., Nash, T.W., Horwitz, M.A. (1986) Interferon-gamma activated human monocytes inhibit the intracellular multiplication of *Legionella pneumophila.* J. Immunol. **137:** 2662-2669.

Biberstein, E.L. (1981) "*Haemophilus somnus*" and "*Haemophilus agni*" in "*Haemophilus Pasteurella* and *Actinobacillus*", edited by M. Kilian, W. Frederiksen and E.L. Biberstein. Academic Press, London pp. 125-132.

Black, C., Bermudez, L.E., Young, L.S., and Remington, J. (1990) Co-infection of macrophages modulates IFN-γ and TNF-induced activation against intracellular pathogens. J. Exp.Med. **172:** 977-982.

Blackwell, J.M., Roberts, B., Alexander, J., (1985) Response of BALB/c mice to leishmanial infection. Curr. Top. Microbiol. Immunol. **122:** 97-106.

Blackwell, J., Ezekowitz, R.A.B., Roberts, M.B., Channon, J.Y., Sim, R.B., and Gordon, S. (1985b) Macrophage complement and lectin-like receptors bind *Leishmania* in the absence of serum. J. Exp. Med. **162:** 324-331.

Blanchard, D.K., Djeu, J.Y., Klein, T.W., Friedman, H., and Steward, W.E. (1988) Protective effect of TNF in experimental *Legionella pneumophila* infection of mice via activation of PMN function. J. Leucocyte. Biol. **43:** 429-434.

Bloom, B.R., and Bennet, B. (1966) Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. Science **153:** 80-82.

Blood, D.C., Radostits, and Henderson, J.A. (eds) (1983) *Haemophilus* septicemia of cattle. In Veterinary Medicine - Textbook of the disease of cattle, sheep, pigs, goats, and horses. - 6th edition. Bailliere Tindall, Toronto, ON, p. 625-627.

Blusse, van Oud Ablas A., van der Linden Schrever B., and van Furth . (1983) Origin and kinetics of pulmonary macrophages during an inflammatory reaction induced by intra-alveolar administration of aerosolized heat-killed BCG. Am. Rev. Respir. Dis. **128:** 276-81.

Blusse, van Oud Ablas A., and van Furth R., (1979) Origin, kinetics and characteristics of pulmonary macrophages in the normal steady state. J. Exp. Med. **149:** 1504-1518.

Bogdan, C., Moll, H., Solbach, W., Rollinghoff, M. (1990) Tumor necrosis factor alpha in combination with interferon-gamma, but not with interleukin-4 activates murine macrophages for elimination of *Leishmania major* amastigotes. Eur. J. Immunol. **20:** 1131-1135.

Bradley, D.J., Taylor, B.A., Blackwell, J., Evans, E.P., and Freeman, J. (1979) Regulation of *Leishmania* populations within the host. 3. Mapping of the locus controlling susceptibility to visceral leishmaniasis in mouse. Clin. Exp. Immunol. **37:** 7-14.

Bradley, S.F., Vibhagool, A., Kunkel, S.L., and Kauffman, C.A. (1989) Monokine secretion in aging and protein malnutrition. J. Leucocyte. Biol. **45:** 510-514.

Brakenhoff, J.B.J., De Groot, E.R., Evers, R.F., Pannekoek, H., and Aarden, L.A. (1987) Molecular cloning and expression of hybridoma growth factor in *E. coli*. J. Immunol. **139:** 4116-4121.

Brown, L.N., Dillman, R.C., and Dierks, R.E. (1970) The *Haemophilus somnus* complex. Proc. U.S. Anim. Hlth. Ass. **74:** 94-108.

Brown, I.N., Glynn, A.A., and Plant, J. (1982) Inbred mouse strain resistance to *Mycobacterium lepraemurium* follows the *Ity/Lsh* pattern. Immunology. **47:** 149-156.

Brown, E.J., and Goodwin, J.L. (1988) Fibronectin receptors of phagocytes. Characterization of the Arg-Gly-Asp. binding proteins of human monocytes and polymorphonuclear leucocytes. J. Exp. Med. **167:** 777-793.

Brunt, L.M., Portnoy, D.A., and Unanue, E.R. (1990) Presentation of *Listeria monocytogenes* to CD8 T cells requires secretion of haemolysin and intracellular bacterial growth. J. Immunol. **145:** 3540-3546.

Bryson, D.C., Ball, H.J., McAliskey, M., McConnell, W., and McCullough, S.J. (1990) Pathological, immunohistochemical and microbiological findings in calf pneumonias associated with *Haemophilus somnus* infection J. Comp. Path. **103:** 433-445.

Buchmeier, N.A., and Heffron, F. (1989) Intracellular survival of wild type *Salmonella typhimurium* and macrophage sensitive mutants in diverse populations of macrophages. Infect. Immun. **57**: 1-7.

Buchmueller-Rouiller, Y., and Manuel, J. (1987) Impairment of oxidative metabolism of mouse peritoneal macrophages by intracellular *Leishmania* sp. Infect. Immun. **55**: 587-593.

Bullock, W.E., and Wright, S.D. (1987) Role of the adherence promoting receptors, CR3, LFA-1 and p150,95, in binding of *Histoplasma capsulatum* by human macrophages. J. Exp. Med. **165**: 195-210.

Burchett, S., Weaver, W., Westall, J., Larsen, A., Kronhein, S., and Wilson, C. (1988) Regulation of tumor necrosis factor/cachectin and IL-1 secretion in human mononuclear phagocytes. J. Immunol. **140**: 3473-3481.

Byrd, T.F., Horwitz, M.A. (1987) Intracellular multiplication of *Legionella pneumophila* in human monocytes is iron dependant and the capacity of activated monocytes to inhibit intracellular multiplication is reversed by iron-transferrin. Clin. Res. **35**: 613A.

Byrne, G.I., Lehmann, L.K., Landry, G.J. (1986) Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular *Chlamydia psittaci*. Infect. Immun. **53**: 347-351.

Cain, D.V.(1985) Perinatal calf mortality in the Kansas Flint Hills. MSc Thesis, Kansas State University, Manhattan, Kansas.

Cain, D.V., and Dennis, S.M. (1987) Perinatal calf mortality. Agri-Pract **8**: 11-16.

Cain, J.A., Newmann, S.L., Ross, G.D., (1988) Role of complement receptor type three and serum opsonins in the neutrophil response to yeast. Complement **4**: 75-86.

Canto, J., Biberstein, E.L., Schulte, T.A., and Behymer, D. (1983) Cross-reactivity of *Haemophilus somnus* antibody in agglutination and complement fixation tests and in the enzyme-linked immunosorbant assay. J. Clin. Microbiol. **17(3)**: 500-506.

Carlin, J.M., Ozaki, Y., Bryne, G.I., Brown, R.R., and Borden, E.C. (1989) Interferons and indolamine 2,3-dioxygenase: role in antimicrobial and antitumour effects. Experientia **45**: 535-541.

Carrol, M.E., Jackett, P.S., Aber, V.R., and Lowrie, D.B. (1979) Phagolysosome formation, cyclic adenosine 3':5' monophosphate and the fate of *Salmonella typhimurium* within mouse peritoneal macrophages. J. Gen. Microbiol. **110**: 421-429.

Castell, J.V., Gomez-Lection, M.J., David, M., Andus, T., Geiger, T., Trullenque, R., Fabra, R., and Heinrich, P.C. (1989) Interleukin 6 is the major regulator of the acute phase protein synthesis in adult human hepatocytes. FEBS Lett. **242**: 237-239.

Castell, J., Gomez-Lechon, M., David, M., Hirano, T., Kishimoto, T., and Heinrich, P. (1988) Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes. FEBS Lett. **232**: 347-350.

Cavanaugh, D.C. and Randall, R. (1959) The role of multiplication of *Pasteurella pestis* in mononuclear phagocytes in the pathogenesis of flea-born plague. J. Immunol. **83**: 348-363.

Chan, J., Fan, X., Hunter, S.W., Brennan, P.J., and Bloom, B.R. (1991) Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infect. Immun. **59**: 1755-1761.

Chan, J., Fujiwara, T., Brennan, P., McNeil, M., Turco, S.J., Sibille, J.C., Snapper, Aisen, P., and Bloom, B.R. (1989) Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc. Natl. Acad. Svi. USA **86**: 2453-2457.

Chandler, D.B., and Fulmer, J.D. (1987) Prostaglandin synthesis and release by subpopulations of rat alveolar macrophages. J. Immunol. **139**: 893-898.

Chandler, D.B., Fuller, W.C., Jackson, R.M., and Fulmer, J.D. (1986) Studies of membrane receptors and phagocytosis in subpopulations of rat alveolar macrophages. Am. Rev. Respir. Dis. **133**: 461-467.

Chang, K.P. (1979) *Leishmania donovani*: promastigote-macrophage surface interactions *in vitro*. Exp. Parasitol. **48**: 175-189.

Chang, K.P. and Dwyer, D.M. (1976) Multiplication of a human parasite (Leishmania donovani) in phagolysosomes of hamster macrophages *in vitro*. Science **193**: 678-680.

Chang, K.P., and Dwyer, D.M. (1978) *Leishmania donovani;* Hamster macrophage interactions *in vitro*: cell entry, intracellular survival, and multiplication of amastigotes. J. Exp. Med. **147**: 515-530.

Channon, J.Y., Roberts, M.B., and Blackwell, J.M. (1984) A study of the differential respiratory burst activity elicited by promastigotes and amastigotes of *Leishmania donovani* in murine residential peritoneal macrophages. Immunology **53**: 345-355

Charnetzky, W.T., and Shuford, W.W. (1985) Survival and growth of *Yersinia pestis* with in macrophages and an effect on the loss of the 47-megadalton plasmid on growth in macrophages. Infect. Immun. **47:** 234-241.

Chatterjee, D., Roberts, A.D., Lowell, K., Brennan, P.J., and Orme, I.M. (1992a) Structural basis of capacity of lipoarabinomannan to induce secretion of tumor necrosis factor. Infect. Immun. **60:** 1249-1253.

Chatterjee, D., Lowell, K., Rivoire, B., McNeil, M.R., and Brennan, P.J. (1992b) Lipoarabinomannan of *Mycobacterium tuberculosis*: capping with mannosyl residues in some strains. J. Biol. Chem. **267:** 6234-6239.

Chatterjee, D., Hunter, S.W., McNeil, M., and Brennan, P.J. (1992c) Lipoarabinomannan; multiglycosylated form of mycobacterial mannosyl phosphatidylinositols. J. Biol. Chem. **267:** 6228-6233.

Chelmonska, A. (1990) The influence of *Haemophilus somnus* on bull sperms examined in vitro. Pol. Arch. Weterynar. **30:** 141-153.

Chen, L., Suzuki, Y., and Wheelock, E.F. (1987) Interferon-γ synergies with tumor necrosis factor and with interleukin-1 and requires the presence of both monokines to induce antitumour cytotoxic activity in macrophages. J. Immunol. **12:** 4096-4101.

Chiang, Y.W., Kaeberle, M.L., and Roth, J.A. (1986) Identification of suppressive components in *Haemophilus somnus* fractions which inhibit bovine polymorphonuclear leucocyte function. Infect. Immun. **52(3):** 792-797.

Chiang, Y.W., Roth, J.A., and Andrews, J.J. (1990) Influence of recombinant bovine interferon gamma and dexamethasone on pneumonia attributable to *Haemophilus somnus* in calves. Am.J.Vet.Res. **51:** 759-762.

Clark, I.A., Hunt, N.H., and Cowden, W.B. (1986) Oxygen derived free radicals in the pathogenesis of parasitic diseases. Adv. Parasitol. **25:** 1-44.

Clerc, P.L., Ryter, A., Mounier, J., and Sansonetti, P.J. (1987) Plasmid mediated killing of eukaryotic cells by *Shigella flexineri* as studied by infection of J774 macrophage. Infect. Immun. **55:** 521-527.

Closs, O., and Haugen, O.A. (1975) Experimental murine leprosy. 3. Early local infection to *Mycobacterium lepraemurium* in C3H and C57BL mice. Acta. Pathol. Microbial. Scand. Sect. **A 83:** 51-58.

Collier, J., and Vallance, P. (1989) Second messenger role for NO widens to nervous and immune system. Trends. Pharmacol. Sci. **123:** 427-31.

Corbeil, L.B. (1990) Molecular aspects of some virulence factors of *Haemophilus somnus*. Can. J. Vet. Res. **54(supplement)**: s57-s62.

Corbeil, L.B., Arthur, J.E., Widders, P.R., Smith, J.W., and Barbet, A.F. (1987) Antigenic specificity of convalescent serum from cattle with *Haemophilus somnus* induced experimental abortion. Infect. Immun. **55(6)**: 1381-1386.

Corbeil, L.B., Blau, K., Prieur, D.J., and Ward, A.C.S. (1985) Serum susceptibility of *Haemophilus somnus* from bovine clinical cases and carriers. J. Clin. Microbiol. **22(2)**: 192-198.

Corbeil, L.B., Widders, P.R., Gogolewski, R., Arthur, J., Inzana, T.J.. and Ward, A.C.S. (1986) *Haemophilus somnus:* Bovine reproductive and respiratory disease. Can. Vet. J. **26**: 90-93.

Corboz, L. (1981) Epidemiology of "*Haemophilus somnus*" infection in cattle: clonal variants of strains isolated from various sources. in: Kilian, M., Frederiksen, W., and Biberstein, E.L. *Haemophilus, Pasteurella, and Actinobacillus.* Academic Press, Toronto, ON. p.133-142.

Corrollo, M.E.W., Jackett, P.S., Aber, V.R., and Lowrie, D.B. (1979) Phagolysosomal formation, cyclic adenosine 3':5' -monophosphate and the fate of *Salmonella typhimurium* within mouse peritoneal macrophages. J. Gen. Microbiol. **110**: 421-429.

Corstvet, R.E., Panciera, R.J., Rinker, H.B., Starks, B.L., and Howard, C. (1973) Survey of tracheas of feedlot cattle for *Haemophilus somnus* and other selected bacteria. J. Am. Vet. Med. Assoc. **163**: 870-873.

Cox, G.W., Melillo, G., Chattopadhyay, U., Mullet, D., Fertel, R.H., and Varesio, L. (1992) Tumor necrosis factor-α-dependent production of reactive nitrogen intermediates mediates IFN-gamma plus IL-2-induced murine macrophage tumoricidal activity. J. Immunol. **149**: 3290-3296.

Crandell, R.A., Smith, A.R., and Kissil, M. (1977) Colonization and transmission of *Haemophilus somnus* in cattle. Am. J. Vet. Res. **38**: 1749-1751.

Crowle, A.J., Dahl, R., Ross, E., and May, M.H. (1991) Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in cultured human macrophages are not acidic. Infect. Immun. **59**: 1823-1831.

Czuprynski, C.J., and Hamilton, H. (1985) Bovine neutrophils ingest but do not kill *Haemophilus somnus in vitro*. Infect. Immun. **50(2)**: 431-436.

David, J.R., (1966) Delayed hypersensitivity *in vitro*: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. Proc. Natl. Acad. Sci. USA **65**: 72-77.

Dees, C., Fountain, M.W., Taylor, J.R., and Schultz, R.D. (1985) Enhanced intraphagocytic killing of *Brucella abortus* in bovine mononuclear cells by liposomes-containing gentamycin. Vet. Immunol. Immunopathol. 8: 171-182.

Denis, M. (1991) Modulation of *Mycobacterium lepraemurium* growth in murine macrophages: Beneficial effect of Tumor necrosis factor alpha and Granulocyte-Macrophage Colony-Stimulating factor. Infect. Immun. **59(2)**: 705-707.

Denis, M., Forget, A., Pelletier, M., Gervais, F., and Skamene, E. (1990) Killing of *Mycobacterium smegmatis* by macrophages from BCG-susceptible and BCG-resistance strains of mice. J. Leucocyte. Biol. **47**: 25-31.

Densen, P., and Mandell, G.L. (1980) Phagocyte strategy vs microbial tactics. Rev. Infect. Dis. **2**: 817-838.

Desiderio, J.V., Kiener, P.A., Lin, P.F., and Warr, G.A. (1989) Protection of mice against *Listeria monocytogenes* infection by recombinant tumor necrosis factor alpha. Infect. Immun. **57**: 1615-1617.

Detmers, P.A., and Wright, S.D. (1988) Adhesion promoting receptors on leucocytes. Curr. Opin. Immunol. **1**: 10-15.

Deuren, M.V., Dofferhoff, A.S.M., and van der Meer, J.W.M. (1992) Cytokines and the response to infection. J. Pathol. **168**: 349-356.

Dewey, K.J. and Little, P.B. (1984) Environmental survival of *Haemophilus somnus* and influence of secretions and excretions. Can, J. Comp. Med. **48**: 23-26.

Dike, L., and Farmer, S. (1988) Cell adhesion induces expression of growth-associated genes in suspension -arrested fibroblasts. Proc. Natl. Acad. Sci. USA **85**: 6792-6796.

Dillman, R.C. (1972) Laryngitis in feedlot calves and its association with the *Haemophilus somnus* (Somnifer) complex. Proc. US Anim Health Assoc **76**: 498-501.

Ding, A.H., Nathan, C.F., and Stueher, D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. J. Immunol. **141**: 2407-2413.

Djeu, J.Y., Blanchard, D.K., Halkias, D., and Friedman, H. (1986) Growth inhibition of *Candida albicans* by human polymorphonuclear neutrophils: activation by IFN-γ and tumor necrosis factor. J. Immunol. **137:** 2980-2986.

Dommisse, G.F. (1975) The arteries and veins of the human spinal cord from birth. Edinburgh: Churchill Livingstone, 81-96.

Donnelly, C.W., and Baiegant, G.J. (1986) Method for flow cytometric detection of *Listeria monocytogenes* in milk. Appl. Environ. Microbiol. **52:** 689-695.

Douvas, G.S., Looker, D.L., Vatter, A.E., and Crowle, A.J. (1985) γ-Interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria. Infect. Immun. **50:** 1-8.

Drapier, J.C., and Hibbs, Jr. J.B. (1988) Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulpher enzymes in the macrophage effector cells. J. Immunol. **140:** 2829-2838.

Drath, D.B., Karnovsky, M.L. (1975) Superoxide production by phagocytic leucocytes. J. Exp. Med. **141:** 257-61.

Dyer, R.M., Benson, C.E. and Boy, M.G. (1985) Production of superoxide anion by bovine pulmonary macrophages challenged with soluble and particulate stimuli. Am. J. Vet. Res. **46:** 336-341.

Dyer, R.M., Erney, S., Spencer, P. and Benson, C.E. (1989) Oxidative metabolism of bovine alveolar macrophages. Am. J. Vet. Res. **50(4):** 448-454.

Easmon, C.S.F., Cole, P.J., Williams, A.J., *et al.* (1980) The measurement of opsonic and phagocytic by luminol-dependent chemiluminescence. Immunology **41:** 67-74.

Eissenberg, L.G., and Goldman, W.E. (1987) *Histoplasma capsulatum* fails to trigger the release of superoxide from macrophages. Infect. Immun. **55:** 29-34.

Eissenberg, L.G., Wyrick, P.B., Davis, C.H., Rump, J.W. (1983) *Chlamydia psittaci* elementary body envelops: ingestion and inhibition of phagolysosomal fusion. Infect. Immun. **40:** 741-751.

Ellis, J.A., Godson, D., Campos, M., Sileghem, M., and Babiuk, L.A. (1993) Capture immunoassay for ruminant tumor necrosis factor-a: comparison with bioassay. Vet. Immunol. Immunopathol. **35:** 289-300.

English, D., Roloff, J.S. and Lukens J.N. (1981) Regulation of human polymorphonuclear leucocyte superoxide release by cellular responses to chemotactic peptides. J. Immunol. **126**: 165-171.

Espinoza-Delgado, I., Longo, D.L., Gusella, G.L., and Varesio, L. (1992) Regulation of IL-2 receptor subunit genes in human monocytes: Differential effect of IL-2 and IFN-γ. J. Immunol. **149**: 2961-2968.

Everson, M.P., Brown, C.B., and Lilly, M.B. (1989) Interleukin-6 and granulocyte-macrophage colony-stimulating factor are candidate growth factor for chronic myelomonocytic leukemia cells. Blood **74**: 1472-1476.

Ezekowitz, R.A., Sim, R.B., Hill, M., and Gordon, S. (1984) Local opsonization by secreted macrophage complement components. Role of receptors for complement in uptake of zymosan. J. Exp. Med. **159**: 244-260.

Fantone, J.C., and Ward, P.A. (1982) Role of oxygen-derived free radicals and metabolites in leucocyte-dependant inflammatory reactions. Am. J. Pathol. **107**: 397-418.

Fels, A.O.S., Nathan, C.F., Cohn, Z.A. (1987) Hydrogen peroxide release by alveolar macrophages from sarcoid patients and by alveolar macrophages from normals after exposure to recombinant interferons alpha, beta, and gamma and 1,25-dihydroxyvitamin D. J. Clin. Invest. **80**: 381-7.

Fenwick, B.W. (1990) Virulence attributes of the lipopolysaccharides of the HAP group organisms. Can. J. Vet. Res. **54(supplement)**: s28-s32.

Fields, P.I., Groisman, E.A., and Heffron, F. (1989) A *Salmonella* locus that controls resistance to microbial proteins from phagocytic cells. Science. **243**: 1059-1062.

Fields, P.I., Swanson, R.V., Haidaris, C.G., and Heffron, F. (1986) Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are virulent. Proc. Natl. Acad. Sci. USA **83**: 5189-5198.

Flesch, I., and Kaufmann, S.H.E. (1987) Mycobacterial growth inhibition by inteferon-γ activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. J. Immunol. **138**: 4408-4413.

Flesch, I.A., and Kaufmann, S.H.E. (1988) Attempts to characterize the mechanisms involved in mycobacterial growth inhibition by gamma-interferon-activated bone marrow macrophages. Infect. Immun. **56**: 1464-1469.

Frasca, D., Adorini, L., Landolfo, S., Doria, G. (1985) Enhancing effect of IFN-γ on helper T cell activity and IL-2 production. J. Immunol. **134**: 3907-3911.

Friis, R.R. (1972) Interaction of L cells and Chlamydia psittaci: entry of the parasite and host responses to its development. J. Bacteriol. **110**: 706-721.

Furness, G. (1958) Interaction between *Salmonella typhimurium* and phagocytic cells in tissue culture. J. Infect. Dis. **103**: 273-277.

Gaillard, J.L., Berche, P. , and Sansonetti, P. (1986) Transposon mutagenesis as a tool to study the role of haemolysin in the virulence of Listeria monocytogenes. Infect. Immun. **52**: 50-55.

Gaillard, J.L., Berche, P., Sansonetti, P. (1987) Transposon mutagenesis as a tool to study the role of haemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. **55**: 2822-2829.

Garcia-Delgado, G.A., Little, P.B., and Barnum, D.A. (1977) A comparison of various *Haemophilus somnus* strains. Can. J. Comp. Med. **41**: 380-388.

Gauldie, J., Richards, C., Harnish, D., Lansdorp, P., and Baumann, H. (1987) Interferon β2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc. Natl. Acad. Sci. USA **84**: 7251-7255.

Gazzinelli, R.T., Oswarld, I.P., James, S.L., and Sher, A. (1992) IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma activated macrophages. J. Immunol. **148**: 1792-1796.

Geiger, T., Andus, T., Klapproth, J., Hirano, T., Kishimoto, T., and Heinrich, P.C. (1988) Induction of rat acute-phase proteins by interleukin 6 *in vivo*. Eur. J. Immunol. **18**: 717.

Gendalman, H.E., Orenstein, J.M., Martin, M.A., Ferrua, C., Mitra, R., Phipps, T., Wahl, L.A., Lane, H.C., Fauci, A.S., Burke, D.D., Skillman, D., and Meltzer, M.S. (1988) Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1 treated monocytes. J. Exp. Med. **167**: 1428-1441.

Glossing, J. (1966) The bacteria isolated from lesions of embolic meningoencephalitis of cattle. Illinois Vet. **9(3)**: 14-18.

Godleski, J., and Brain, J.D., (1972) The origin of alveolar macrophages in mouse radiation chimeras. J. Exp. Med. **136**: 630-43.

Gogolewski, R.P., Kania, S.A., Inzana, T.J., Widders, P.R., Liggitt, H.D., and Corbeil, L.B. (1987b) Protective ability and specificity of convalescent serum from calves with *Haemophilus somnus* pneumonia. Infect. Immun. **55(6)**: 1403-1411.

Gogolewski, R.P., Leathers, C.W., Liggitt, H.D., and Corbeil, L.B. (1987a) Experimental *Haemophilus somnus* pneumonia in calves and immunoperoxidase localization of bacteria. Vet. Pathol. **24**: 250-256.

Gogolewski, R.P., Schaefer, D.C., Wasson, S.K., Corbeil, R.R., and Corbeil, L.B. (1989) Pulmonary persistence of *Haemophilus somnus* in the presence of specific antibody. J. Clin. Microbiol. **27(8)**: 1767-1774.

Gomis, S. M., Van Donkersgoed, J., Harland, R.J., Potter, A.A and Haines, D.M. Immunohistochemical demonstration of the distribution of *H. somnus* in feedlot calves after an experimental infection. (1993) Abstract # 188, Vet. Pathol. 30(5): 474.

Granger, D.L., Hibbs, Jr. J.B., Perfect, J.R., and Durack, D.T. (1990) Metabolic fate of L-arginine in relation to microbiostatic capacity of murine macrophages. J. Clin. Invest. **85**: 264-273.

Granger, D.L., Perfect, J.R., and Durack, D.T. (1986) Macrophage mediated fungistasis: requirement for a macromolecular component in serum. J. Immunol. **137**: 693-701.

Grebner, J.V., Mills, E.L., and Gray, B.H. (1977) Comparison of phagocytic and chemiluminescence response of human polymorphonuclear neutrophils. J. Lab. Clin. Med. 89: 153-159.

Green, S.J., Crawford, R.M., Hockmeyer, J.T., Meltzer, M.S., and Nacy, C.A. (1990) *Leishmania major amastogotes* initiate the L-arginine-dependent killing mechanism in IFN-γ -stimulated macrophages by induction of tumor necrosis factor-α. J. Immunol. **145**: 4290-4297.

Green, S.J., Nacy, C.A., and Meltzer, M.S. (1991) Cytokine induced synthesis of nitrogen oxides in macrophages: a protective host response to *Leishmania* and other intracellular pathogens. J. Leucocyte. Biol. **50**: 93-103.

Griner, L.A., Jensen, R., and Brown, W.W. (1956) Infectious embolic meningoencephalitis in cattle. J. Am. Vet. Med. Assoc. **129**: 417-421.

Groom, S.C. (1985) Guelph case control study. MSc Thesis, University of Guelph, Guelph, Ontario.

Groom, S.C., Little, P.B., and Rosendal, S. (1988) Virulence differences among three strains of *Haemophilus somnus* following intratrachial inoculation of calves. Can. J. Vet. Res. **52**: 349-354.

Haines, D.M., and Clark, E.G. (1991) Enzyme immunohistochemical staining of formalin-fixed tissues for diagnosis in veterinary pathology. Can.Vet. J. **32**: 295-301.

Hall, R.F., Williams, J.M., and Smith, G.L. (1977) Field evaluation of *Haemophilus somnus* bacterin. Vet. Med. Small. Anim. Clin. **72**: 1368-1370.

Hance, A.J., Douches, S., Winchester, R.J., Ferrans, V.J., Crystal, R.G. (1985) Characterization of mononuclear phagocyte subpopulations in the human lung by using monoclonal antibodies: changes in alveolar macrophage phenotype associated with sarcoidosis. J. Immunol. **134**: 284-92.

Hansen, M.B., Nielsen, S.E., and Berg, K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/kill. J. Immunol. Methods, **119**: 203-210.

Harland, R.J., Potter, A.A., and Schuh, J.C.L. (1990) Development of an intravenous challenge model for *Haemophilus somnus* disease in beef calves. 71st Conf. Res. Work. Anim. Dis. **29**: 6.

Harris, F.W. and Janzen, E.D. (1989) The *Haemophilus somnus* disease complex (Haemophilosis): A review. Can. Vet. J. **30**: 816-822.

Harris, S.E., Nelson, S., Astry, C., Bainton, B.G., and Summer, W.R. (1988) Endotoxin induced suppression of pulmonary antibacterial defence against *Staphylococcus aureus*. Am. Rev. Respir. Dis. **138**: 1439-1443.

Hasui, M., Hirabayashi, Y., and Kobayashi, Y. (1989) Simultaneous measurement by flow cytometry of phagocytosis and hydrogen peroxide production of neutrophils in whole blood. J. Immunol. Methods **117**: 53-58.

Havell, E.A. (1989) Evidence that tumor necrosis factor has an important in antibacterial resistance. J. Immunol. **143**: 2894-2899.

Havell, E.A., and Sehgal, P.B. (1991) Tumor necrosis factor independent IL-6 production during murine listeriosis. J. Immunol. **146**: 756-761.

Haynes, J.L. (1988) Principles of flow cytometry. Cytometry **3**: 7-17

Hazlett, M.J., Little, P.B., and Barnum, D.A. (1983) Experimental production of mastitis with Haemophilus somnus in the lactating bovine mammary gland. Can. Vet. J. 24: 135-136.

Hedges, S., Anderson, P., Lidin-Jansen, G., de Man, P., and Svanborg, C. (1991) Interleukin-6 responses to deliberate colonization of the human urinary tract with gram-negative bacteria. Infect. Immun. **59**: 421-427.

Hetland, G., and Eskeland, T. (1986) Formation of the alternative pathway of complement by human monocytes *in vitro* as demonstrated by phagocytosis of agarose beads. Scand. J. immunol. **23**: 301-308.

Hetland, G., Johnson, E., and Aasebo, U. (1986) Human alveolar macrophages synthesized the functional alternative pathway of complement and active C5 and C9 *in vitro*. Scand. J. immunol. **24**: 603-608.

Hetland, J., Johnson, E., Royset, P., and Eskeland, T. (1987) Human alveolar macrophages and monocytes generate the functional classical pathway of complement *in vitro*. Acta. Pathol. Microbiol. Scand [C]. **95**: 117-121.

Hibbs, J.B., Taintr, R.R., Vavrin, Z., and Rachlin, E.M. (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Comm. **157**: 87-94.

Hibbs, J.B., Taintr, R.R.Jr., and Vavrin, Z. (1987a) Macrophage cytotoxicity: role of L-arginine deiminase and imino nitrogen oxidation to nitrite. Science (Washington D.C.) **235**: 473-476.

Hibbs, J.B., Vavrin, Jr. Z., and Taintor, R.R. (1987b) L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J. Immunol. **138**: 550-565.

Hibbs, J.B. et al., (1990) in Nitric Oxide from L-arginine: a bioregulatory system (Moncada, S. and Higgs, E.A., eds) p. 189-223, Excerpta Medica

Higgins, R., Martin, J.R., Larouche, Y., and Goyette, G. (1987) Mastitis caused by *Haemophilus somnus* in dairy cow. Can. Vet. J. **28**: 117-119.

Hines II, M.E., Jaynes, J.M., Barker, S.A., Newton, J.C., Enright, F.M., and Snider 3, T.G. (1992) Isolation and partial characterizations of glycolipid fractions from *Mycobacterium avium* serovar 2 (*Mycobacterium paratuberculosis* 18) that inhibit activated macrophages. Infect. Immun. **61(1)**: 1-7.

Ho, J.L., Reed, S.G., Sobel, J., Arruda, S., He, S.H., Wick, E.A., and Grabstein, K.H. (1992) Interleukin-3 induces antimicrobial activity against *Leishmania amazonensis* and *Trypanasoma cruzi* and tumoricidal activity in human peripheral blood-derived macrophages. Infect. Immun. **60**: 1984-1993.

Ho, J.L., Reed, S.G., Wick, E.A., and Giordano, M. (1990) Granulocyte macrophage and macrophage colony stimulating factors activate intramacrophage killing of *Leishmania amasonensis*. J. Infect. Dis. **157**: 9250930.

Hoffmann, R.A., Langrehr, J.M., Billiar, T.R., Curran, R.D., and Simmons, R.L. (1990) Allo-antigen-induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. J. Immunol. **145:** 2220-2226.

Holian, A., Dauber, J., Diamond, M.S., and Daniele, R.P. (1983) Separation of bronchoalveolar cells from the guinea pig on continuous density gradient of Percoll: functional properties of fractionated lung macrophages. J. Reticuloendothel. Soc. **33:** 157-64.

Holzer, T.J., Nelson, K.E., Schauf, V., Crispen, R.G. and Anderson, B.R. (1986) *Mycobacterium leprae* fails to stimulate phagocytic cell superoxide generation. Infect. Immun. **51:** 514-520.

Hoover, D.L., Nacy, C.A., and Meltzer, M.S. (1985) Human monocyte activation for cytotoxicity against intracellular *Leishmania donovani* amastigotes: induction of microbicidal activity by interferon gamma. Cell. Immunol. **94:** 500-511.

Horan, P.K., Melinicoff, M.J., Jansen, B.D., and Slezak, S.E. (1990) Fluorescent cell labelling for *in vivo* and *in vitro* cell tracking. Methods. Cell. Biol. **33:** 469-490.

Horan, P.K., and Slezak, S.E. (1989) Stable cell membrane labelling. Nature(London) **340:** 167-168.

Hormaeche, C.E. (1980) The *in vivo* division and death rates of *Salmonella typhimurium* in the spleens of naturally resistance and susceptible mice measured by the superinfection phage technique of Meynell. Immunology **41:** 973-979.

Horwitz, M.A. (1983a) Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila*) in human monocytes. J. Exp. Med. **158:** 1319-1331.

Horwitz, M. A. (1983b) The legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158:** 2108-2126.

Horwitz, M.A. (1984) Phagocytosis of Legionnaires' disease bacterium (*Legionella pneumophila*) occur by novel mechanism; engulfment within pseudopod coil. Cell. **36:** 27-33.

Horwitz, M.A., (1988) Intracellular parasitism. Curr. Opin. Immunol. **1:** 41-46.

Horwitz, M.A., and Maxfield, F.R. (1984) *Legionella pneumophila* inhibit acidification of its phagosome in human monocytes. J. Cell. Biol. **99:** 1936-1943.

Hubbard, R.D., Kaeberle, M.L., Roth, J.A., and Chiang, Y.W. (1986) *Haemophilus somnus* - induced interference with bovine neutrophil functions. Vet. Microbiol. **12**: 77-85.

Humphrey, J.D. (1982) *Haemophilus somnus*: colonization of the bovine reproductive tract, strain variation, and pathogenicity. Ph.D. thesis. University of Guelph, Guelph, ON, pp. 298.

Humphrey, J.D., Little, P.B., Stephens, L.R., Barnum, D.A., Doig, P.A., and ThorsenJ. (1982a) Prevalence and distribution of *Haemophilus somnus* in the male bovine reproductive tract. Am. J. Vet. Res. **43**: 791-795.

Humphrey, J.D., Little, P.B., Barnum, D.A., Doig, P.A. Stephens, L.R., and Thorsen, J. (1982b) Occurrence of *Haemophilus somnus* in bovine semen and in the prepuce bulls and steers. Can. J. Comp. Med. **46**: 215-217.

Humphrey, J.D., and Stephens, L.R. (1983) '*Haemophilus somnus*' : a review. Vet. Bull. **53(11)**: 987-1004.

Hunter, S.W., and Brennan, P.J. (1981) A novel phenolic glycolipid from *Mycobacterium leprae* possibly involved in immunogenicity and pathogenicity. J. Bacteriol. **147**: 728-735.

Hunter, S.W., Fujwara, T., and Brennan, J. (1982) Structure and antigenicity of the major specific glycolipid antigen of *Mycobacterium leprae*. J. Biol. Chem. **257**: 15072-15078.

Ignaroo, L.J. (1991) Signal transduction mechanisms involving nitric oxide. Biochem. Pharmacol. **41**: 485-490.

Inzana, T.J., and Corbeil, L.B. (1987) Development of a define medium for *Haemophilus somnus* isolated from cattle. Am. J. Vet. Res. **48(3)**: 366-369.

Inzana, T.J., Gogolewski, R.P., and Corbeil, L.B. (1992) Phenotypic phase variation in *Haemophilus somnus* lipooligosaccharide during bovine pneumonia and after *in vitro* passage. Infect. Immun. **60(7)**: 2943-2951.

Inzana, T.J., Iritana, B., Gogolewski, R.P., Kania, S.A., and Corbeil, L.C. (1988) Purification and characterization of lipooligosaccharides from four strains of "*Haemophilus somnus*". Infect. Immun. **56(11)**: 2830-2837.

Isberg, R.R.,and Leong, J.M. (1990) Multiple β1 chain intergrins are receptors for invasion, a protein that promotes bacterial penetration into mammalian cells. Cell. **60**: 861-871.

Isobe, K-I., and Nakashima, I. (1992) Feedback suppression of Staphylococcal enterotoxin-stimulated T-lymphocyte proliferation by macrophages through inductive nitric oxide synthesis. Infect. Immun. **60(11)**: 4832-4837.

Ito, M., Karmali, R., and Krim, M. (1985) Effect of interferon on chemiluminescence and hydroxyl radical production in murine macrophages stimulated by PMA. Immunology **56**: 533-541.

Iwamoto, G.K., Monick, M.M., Clark, B.D., Auron, P.E., Stinski, M.F., and Hunninghake, G.W. (1990) Modulation of interleukin-1 beta gene expression by the immediate early gene of human cytomegalovirus. J. Clin. Invest. **85**: 1853-1857.

Jackett, P.S., Aber, V.R., and Lowrie, D.B. (1978) Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *Mycobacterium tuberculosis*. J. Gen. Microbiol. **104**: 37-

Jackson, J.A., Andrews, J.J., and Hargis, J.W. (1987) Experimental *Haemophilus somnus* pneumonia in calves. Vet. Pathol. **24**: 129-134.

Jacobs R.F., Locksley, R.M., Wilson, C.B., Hass, J.E., and Klebanoff, S.J. (1984) Interaction of primate alveolar macrophages and Legionella pneumophila. J. Clin. Invest. 73: 1515-1523.

James, S.L., and Hibbs, Jr. J.B. (1990) The role of nitrogen oxides as effector molecules parasitic killing. Parasitol. Today **6**: 303-305.

Janzen, E.D., Cates, W.F., and Barth, A, *et al.* Prevalence of *Haemophilus somnus* in the semen of bulls in Saskatchewan. (1981) Can. Vet. J. **22**: 361-362.

Johnson, J., Myerick, B., Jesmok, G., and Brigham, K.L. (1989) Human recombinant tumor necrosis factor alpha infusion mimics endotoxemia in awake sheep. J. Appl. Physiol. **66**: 1448-1454.

Johnston, R.B., Keele, B.B., Misra, H.P., Lehmeyer, J.E., Web, L.S., Baehner, R.L., and Rajogopalan, K.V. (1975) The role of superoxide anion generation in phagocytic bactericidal activity; studies with normal and chronic granulomatous disease leucocytes. J. Clin. Invest. **55**: 1357-1372.

Joiner, K.A., Fuhrman, S.A., Miettinen, H.M., Kasper, L.H., and Mellman, I. (1990) *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. Science **249**: 641-646.

Jones, T.C. and Hirsch, J.G. (1972) The interaction between Toxoplasma gondii and mammalian cells. II The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. J. Exp. Med. **136**: 1173-1194.

Jones, T.C., Yeh, S., and Hirsch, J.G. (1972) The interaction between Toxoplasma gondii and mammalian cells. I Mechanism of entry and intracellular fate of the parasite. J. Exp. Med. **136**: 1157-1172.

Kagaya, K., Watanabe, K., and Fukazawa, Y. (1989) Capacity of recombinant gamma interferon to activate macrophage for *Salmonella* killing activity. Infect. immun. 57: 609-615.

Kato, K., Yokoi, N., Takano, N., Kanegane, H., Yachie, A., Miyawaki, T., and Taniguchi, N. (1990) Detection by in situ hybridization and phenotypic characterization of cells expressing IL-6 mRNA in human stimulated blood. J. Immunol. **144**: 1317-1322.

Kaufmann, S.H., (1988) Immunity against intracellular bacteria: biological effecter functions and antigen specificity of T lymphocytes. Curr. Top. Microbial. Immunol. **138**: 141-176.

Kaufmann, S.H.E. (1989) *In vitro* analysis of the cellular mechanisms involved in immunity to tuberculosis. Rev. Infect. Dis. **11**: 5448-5453.

Kaufmann, S.H.E., and Reddehase, M.J. (1989) Infection of phagocytic cells. Curr. Opin. Immunol. **2**: 43-49.

Kelley, V.E., Fiers, W., and Strom, T.B. (1984) Cloned interferon gamma, but not interferon-β or -α, induces expression of HLA-DR determinants by fetal monocytes and myeloid leukaemic cell lines. J. Immunol. **132**: 240-245.

Kennedy, P.C., Biberstein, E.L., Howarth, J.A., Frazier, L.M., and Dungwoth, D.L. (1960) Infectious meningo-encephalitis in cattle, caused by a *Haemophilus* like organism. Am. J. Vet. Res. **21**: 403-409.

Kilbourn, R.G., and Belloni, P. (1990) Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1 or endotoxin. J. Natl. Cancer Inst. **82**: 772-776.

Kindler, V., Sappino, A.P., Grau, G.E., Piguet, P.F., and Vassalli, P. (1989) The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell **56**: 731-740.

Kishimoto, T., Taga, T., Matsuda, T., Hibi, M., Suematsu, S., Tang, B., Yawata, H., Hirata, Y., Yamaski, K., and Hirano, T. (1989) Interleukin-6 and its receptor in immune regulation, p. 633. In F.M. Melchers et al., (ed.), Progress in immunology, vol. VII. Proceedings of the 7th International Congress of Immunology. Springer-Verlag KG, Berlin.

Klebanoff, S.J. (1968) Myeloperoxidase-halide-hydrogen peroxide antibacterial system. J. Bacteriol. **95**: 2131-2138.

Klebanoff, S.J. (1980) Oxygen metabolism and toxic properties of phagocytes. Ann. Intern. Med. **93**: 480-489.

Klebanoff, S.J. (1982) Oxygen dependant cytotoxic mechanisms of phagocytes, p. 111-162. In J.I. Gallin and A.S. Fauci(ed.), Advances in host defence mechanisms. Raven Press, New York.

Knowles, R.G., Merrett, M., Salter, M., and Moncada, S. (1990) Differential induction of brain, lung and liver nitric oxide synthesis by endotoxin in the rat. Biochem. J. **270**: 833-836.

Kolb, H. (1992) A pathogenic factor in autoimmunity: nitric oxide. Immunol. Today. **13**: 157-160.

Konig, B and Konig, W. (1993) Induction and suppression of cytokine release (tumor necrosis factor-α; interleukin-6, interleukin-1β) by Escherichia coli pathogenicity factors (adhesions, α-haemolysin) Immunology **78**: 526-533.

Kuhlman, M., Joiner, K., and Ezekowitz, R.A.B. (1989) The human mannose binding protein functions as an opsonin. J. Exp. Med. **169**: 1733-1745.

Kuhn, M., and Goebel, W. (1989) Identification of an extracellular protein of *Listeria monocytogenes*. Infect. Immun. **57**: 55-61.

Kuhn, M., Kathariou, S., and Goebol, W. (1988) Haemolysin supports survival but not entry of the intracellular bacterium *Listeria monocytogenes*. Infect. Immun. **56**: 79-82.

Kung, J.T., Brooks, S.B., Jakway, J.P., Leonard, L.L., and Talmage, D.W. (1977) Suppression of *in vitro* cytotoxic response by macrophages due to induced arginase. J. Exp. Med. **146**: 665-672.

Kwiecien, J.K., and Little, P.B. (1992) Isolation of pathogenic strains of *Haemophilus somnus* from the female bovine reproductive tract. Can. J. Vet. Res. **56**: 127-134.

Lamont, H.H., and Hunt, B.W. (1982) *Haemophilus somnus* and conjunctivitis. Vet. Rec. **111**: 21.

Le, J. and Vilcek, J. (1987) Biology of disease. Tumor necrosis factor and interleukin 1: Cytokines with multiple overlapping biological activities. Lab. Invest. **56**: 234-248.

Lederer, J.A., Brown, J.F. and Czuprynski, C.J. (1986) "*Haemophilus somnus*," a facultative intracellular pathogen of bovine mononuclear phagocytes. Infect. Immun. **55(2)**: 381-387.

Lederer, J.A., Brown, J.F., and Czuprynski, C.J. (1987) *Haemophilus somnus*: A facultative pathogen of bovine mononuclear phagocytes. 67th Annu. Meet. Res. Workers Anim. Dis. 1986.

Li, J.Y., and Chadee, K. (1992) Macrophage cytotoxicity against entamoeba tropozoites is mediated by nitric oxide from L-arginine. J. Immunol. **148**: 3999-4005.

Liew, F.Y., and Cox, F.E.G. (1991) Non-specific defence mechanisms-the role of nitric oxide. Immunoparasitol. Today. **2**: A17-A21.

Liew, F.Y., Lee, Y., Severn, A., Millot, S., Schmidt, J., Salter, M., and Moncada, S. (1991) A possible novel pathway of regulation by murine T helper type-2 (TH-2) of a TH1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. Eur. J. Immunol. **21**: 2489-2494.

Liew, F.Y., Li, Y., and Millot, S. (1990) Tumor necrosis factor-α synergies with IFN-γ in mediating killing of *Leishmania major* through the induction of nitric oxide. J. Immunol. **145**: 4306-4310.

Liew, F.Y., Millott, S., Parkinson, C., Palmer, R.M., Moncada, S. (1990) Macrophage killing of *Leishmania* parasite *in vivo* is mediated by nitric oxide from L-arginine. J. Immunol. **144**: 4794-4797.

Little, P.B. (1986) *Haemophilus somnus* complex: Pathogenesis of the thrombotic meningoencephalitis. Can. Vet. J. **27**: 94-96.

Littman, B.H., Dastvan, F.F., Carlson, P.L., Sanders, K.M. (1989) Regulation of monocyte/macrophage C2 production and HLA-DR expression by IL-4 (BSF-1) and IFN-gamma. J. Immunol. **142**: 520-525.

Locksley, R.H., Frankenhauser, J., and Henderson, W.R. (1985) Alteration of leukotrine release by macrophages ingesting *Toxoplasma gondii*. Proc. Natl. Acad. Sci. USA **82**: 6922-6926.

Lurie, M.B. (1942) The fate of tubercle bacilli ingested by phagocytes from normal and immunized animals. J. Exp. Med. **75**: 247-267.

Lyengar, R., Stuehr, D.J., and Marletta, M.A. (1987) Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. Proc. Natl. Acad. Sci. USA **84**: 6369-6373.

MacDonald, D.W., Christian, R.G., and Chalmers, G.A. (1973) Infectious thromboembolic meningoencephalitis: literature review and occurrence in Alberta 1969-1971. Can. Vet. J. **14**: 57-61.

Mackaness, G.B. (1964) The immunological basis of acquired cellular resistance. J. Exp. Med. **120**: 105-120.

Marcinkiewicz, J., and Chain, B.M. (1993) Differential regulation of cytokine production by nitric oxide. Immunology **80**: 146-159.

Marcinkiewicz, J., Radziszewski, W., and Chain, B.M. (1992) Prostaglandin E_2 differentially regulates the production of IL-2 and IL-3 by murine immune T cells. Folia. Histochem. Cytobiol. **30**: 1-4.

Marinkovic, S., Jahreis, G.P., Wong, G.G., and Baumann, H. (1989) IL-6 modulates the synthesis of a specific set of acute phase plasma proteins *in vivo*. J. Immunol. **142**: 808-812.

Marletta, M.A., Yonn, P.S., Lyengar, R., Leaf, C.D., and Wishnok, J.S. (1988) Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry. **27**: 8706-8711.

Mathison, J.C., Wolfson, E., and Ulevitch, R.J. (1988) Participation of tumor necrosis factor in the mediation of gram-negative bacterial lypopolysacchaide induced injury in rabbits. J. Clin. Invest. 81: 1925-1937.

Mauel, J., Ransijn, A., and Buchmuller-Rouiller, Y. (1991) Killing of *Leishmania* parasites in activated murine macrophages is based on an L-arginine-dependent process that produces nitrogen derivatives. J. Leucocyte Biol. **49**: 73-82.

May, L.T., Ghrayeb, J., Santhanam, U., Tatter, S.B., Tatter, S.B., Sthoeger, Z., Helfgott, D.C., Chiorazzi, N., Grieninger, G., and Sehgal, P.B. (1988) Synthesis and secretion of multiple forms of β2-interferon/B-cell differentiation factor 2/hepatocyte -stimulating factor by human fibroblasts and monocytes. J. Biol. Chem. **263**: 7760-7766.

Mayer, B.K., and Falkinham, 3. J.O. (1986) Superoxide dismutase activity of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. Infect. Immun. **53**: 631-635.

Mc Collough, K.C., and Martinod, S., (1987) Applications for immunomodulators. In: A Seminar in Bovine Immunology. Western Veterinary Conference, 16 Feb. 1987, Las Vegas, Nevada, pp. 30-44.

McPhail, L.C., and Snyderman, R. (1984) Mechanisms of regulating the respiratory burst in leucocytes. Contemp. Top. Immunobiol. **14**: 247-284.

Melillo, G., Cox, G.W., Radzioch, D., and Varesio, L. (1993) Picolinic acid, a catabolite of L-Tryptophan, is a costimulus for the induction of reactive nitrogen intermediate production in murine macrophages. J. Immunol. **150:** 4031-4040.

Mellman, I. (1988) Relationships between structure and functions in the Fc receptor family. Curr Opin Immunol. **1:**16-25.

Mendelman, P.M., and Serfass, D.A. (1988) The penicillin binding proteins of the genus *Haemophilus*. J. Med. Microbiol. **27:** 95-98.

Mengaud, J., Vicente, M-F., Chenevert, J., Pereira, J.M. Geoffroy, C., Gicquel,-Sanzey, B., Baquero, F., Perez-Diaz, J-C., and Cossart, P.(1988) Expression of *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56:** 766-772.

Metzger, Z., Hoffeld, J.T. and Oppenheim, J.J. (1980) Macrophage-mediated suppression. 1. Evidence for participation of both hydrogen peroxide and prostaglandins in suppression murine lymphocyte proliferation. J. Immunol. **124:** 983-988.

Meuwissen, H.J., Rhee, M.S., Rynes, R.J., and Pickering, R.J. (1982) Phagocytosis, chemiluminescence and intracellular killing of fungi by phagocytes from subjects with deficiency of the second component of complement. Int. Arch. Appl. Immun. 68: 22-27.

Michaels, F.H., and Banks, K.L. (1988) Contribution of various host factors to resistance to experimentally induced bacterial endotoxemia in calves. Am. J. Vet. Res. **49:** 557-562.

Miliotis, M.D. (1991) Acridine orange stain for determining intracellular enteropathogens in HeLa cells. Microbiol. **29:** 830-831.

Miller, R.B., Lein, D.H., McEntee, K.E., Hall, C.E., and Shin, S. (1983) *Haemophilus somnus* infection of the reproductive tract of cattle: A review. J. Am. Vet. Med. Assoc. **182:** 1390-1392.

Miller, R.J., Renshaw, H.W., and Evans, J.A. (1975) *Haemophilus somnus* complex: antigenicity and specificity of fractions of *Haemophilus somnus*. Am. J. Vet. Res. **36(8):** 1123-1128.

Momotani, E., Whipple, D.L., Thierman, A.B., and Cheville, N.F. (1988) Role of M cells and macrophages in the entrance of *Mycobacterium paratuberculosis* into domes of ileal Payer's patches in calves. Vet. Pathol. **25:** 131-137.

Moncada, S., Palmer, R.M.J., and Higgs, E.A. (1989) Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. Biochem. Pharmacol. **138:** 1709-1715.

Montarroso, A.M. and Myrvik, Q.N. (1979) Oxidative metabolism of BCG activated alveolar macrophages. J. Reticuloendothel. Soc. **25:** 559-574.

Mor, N., Gorden, M.B., Pabst, M.J. (1989) *Mycobacterium lepraemurium* activates macrophages but fails to trigger release of superoxide anion. J. Immunol. **140:** 3956-3961.

Mosser, D.M., and Edelson, P.J. (1985) The mouse macrophage for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. J. Immunol. **135:** 2785-2789.

Muraguchi, A., Hirano, T., Tang, B., Matsuda, T., Horii, Y., Nakajima, K., and Kishimoto, T. (1988) The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the internal differentiation of B cells. J. Exp. Med. **167:** 332-344.

Murray, H.W., and Cohn, Z.A. (1980) Macrophage oxygen-dependant antimicrobial activity. 3. Enhanced oxidative metabolism as an expression of macrophage activation. J. Exp. Med. **152:** 1596-1609.

Murray, H.W., Rubin, B.Y., Carriero, S.M., Harris, A.M., and Jaffee, F.A. (1985) Human mononuclear phagocyte antiprotozoan mechanisms: oxygen dependant vs oxygen independent activity against intracellular *Toxoplasma gondii.* J.Immunol. **134:** 1982-1988.

Murray, H.W., Rubin, B.Y., and Rothermel, C.D. (1983) Killing of intracellular *Leishmania donovani* by lymphokine-stimulated human phagocytes. Evidence that interferon-gamma is the activating lymphokine. J. Clin. Invest. **72:** 1506-1510.

Murray, H.W., Spitalny, G.L., and Nathan, C.F. (1985b) Activation of mouse peritoneal macrophages *in vitro* and *in vivo* by interferon-γ. J. Immunol. **134:** 1619-1622.

Nacy, C.A., James, S.L., Benjamin, W.R., Farrar, J.J., and Hockmeyer, W.T. and Meltzer, M.S. (1983) Activation of macrophages for microbicidal and tumoricidal effector functions by soluble factors from EL-4, a continuous T cell line. Infect. Immun. **40:** 820-824.

Nacy, C.A., Nelson, B.J., Meltzer, M.S., and Green, S.J. (1992) Cytokines that regulate macrophage production of nitrogen oxides and expression of anti-leishmanial activities. 39th Forum in Immunol. 573.

Najjar, V.A. (1983) Tuftsin, a natural activator of phagocyte cells: an overview. Ann N.Y. Acad. Sci. **419**: 1-11.

Nakajima, Y., and Ueda, H. (1989) Schwartzman reaction in the brain induced by *Haemophilus somnus* and *Escherichia coli* lipopolysaccharide in rabbits. J. Comp. Pathol. **100(3)**: 231-236.

Nakamura, M., Manser, T., Pearson, G.D.N., Daley, M.J., Gefter, M.L. (1984) Effect of IFN-γ on the immune response *in vivo* and on gene expression *in vitro*. Nature (Lond) **307**: 381-382.

Nakane, A., Minagawa, T., and Kato, K. (1988) Endogenous tumor necrosis factor (cachetin) is essential to host resistance against *Listeria monocytogenes* infection. Infect. Immun. **56**: 2563-2569.

Nash, T.W., Libby, D.M., and Horwitz, M.A. (1988) IFN-γ activated human alveolar macrophages inhibit the intracellular multiplication of *Legionella pneumophila*. J. Immunol. **140**: 3978-3981.

Nathan, C.F., and Hibbs, Jr. J.B. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr. Opin. Immunol. **3**: 65-70.

Nathan, C.F., Murray, W.H. and Cohn, Z.A. (1980) The macrophage as an effector cell. New. Engl. J. Med. **303**: 622-626.

Nathan, C.F., Murray, H.W., Wiebe, M.E., and Rubin, B.Y. (1983) Identification of interferon-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. **158**: 670-689.

Nathan, C.F., and Tsunawaki, S. (1984) Enzymatic basis of macrophage activation. J. Biol. Chem. **69**: 4305-4312.

Nation, P.N., Frelier, P.F., Gifford, G.A., and Carnat, B.D. (1983) Otitis in feedlot cattle. Can. Vet. J. **24**: 238.

Neil, M.A., and Klebanoff, S.J. (1988) The effect of phenolic glycolipid-1 from *Mycobacterium leprae* on the antimicrobial activity of human macrophages. J. Exp. Med. **167**: 30-42.

Nicholas, D.J.D., and Nason, A. (1957) Determination of nitrates and nitrites. In: Methods in Enzymology (eds S.F. Colowick and N.O. Kaplan), Vol: 3, p. 981. Academic Press, New York.

Nogueira, N. and Cohn, Z. (1976) Trypanosoma cruzi: Mechanism of entry and intracellular fate in mammalian cells. J. Exp. Med. **143**: 1402-1420.

O'Brien, A.D., Rosenstreich, D.L., and Taylor, B.A. (1980) Control of natural resistance to *Salmonella typhimurium* and *Leishmania donovani* in mice by closely linked but distinct genetic loci. Nature **287:** 440-442.

Ogunnariwo, J.A., Cheng, C., Ford, J., and Schryvers, A.B. (1990) Response of *Haemophilus somnus* iron limitation: expression and identification of a bovine specific transferrin receptor. Micro. Path. **9:** 397-406.

Ohmann, H.B. and Babiuk, L.A. (1984) Effect of bovine recombinant alpha-1 interferon on inflammatory responses of bovine phagocytes. J. Interferon. Res. **4:** 249-263.

Old, L.J. (1985) Tumor necrosis factor (TNF). Science **230:** 630-632.

Orme, M.I., Furney, S.K., Skinner, P.S., Roberts, A.D., Brennan, P.J., Russell, D.G., Shiratsuchi, H., Ellner, J.J., and Weiser, W. (1992) Inhibition of growth of *Mycobacterium avium* in murine and human mononuclear phagocytes by migration inhibitory factor. Infect. Immun. **61(1):** 338-342.

Orr, J.P. (1992) *Haemophilus somnus* infection: A retrospective analysis of cattle necropsied at the Western College of Veterinary Medicine from 1970-1990. Can.Vet.J. **33:** 719-720.

Oswald, I.P., Gazzinelli, R.T., Sher, A. and James, S.L. (1992) IL-10 synergize with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. J. Immunol. **148:** 3578-3582.

Ouaissi, M.A., Afchain, D., Capron, A., and Grimaad, J.A. (1984) Fibronectin receptors on *Trypanosoma cruzi* trypamastigotes and their biological function. Nature **308:** 380-382.

Pabst, M.J., Gross, J.M., Brozna, J.P., and Goren, M.B. (1988) Inhibition of macrophage priming by sulfatide from *Mycobacterium tuberculosis*. J. Immunol. **140:** 636-640.

Pace, J.L., Russel, S.W., Torres, B.A., Johnson, H.M., and Gray, P.W. (1983) Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing. J. Immunol. **130:** 2011-2013.

Palmer, R.M.J., Ashton, D.S., and Moncada, S. (1988) Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature **333:** 664-666.

Palmer, R.M.J., Ferridge, A.G., and Moncada, S. (1987) Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. Nature **327(6122):** 524-526.

Palmer, R.M.J., and Moncada, S. (1989) A novel citrulin-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. Biochem. Biophys. Res. Commun. **158**: 348-352.

Panciera, R.J., Dahlgren, R.R., Rinker, H.B. (1968) Observations on septicemia of cattle caused by a *Haemophilus*-like organism. Pathol. Vet. **5**: 212-226.

Papermaster, B.G., Whitcomb, M.E. and Sagone, A.L. (1980) Characterization of metabolic responses of the human alveolar macrophage. J. Reticuloendothel. Soc. **28**: 129-139.

Passwell, J.H., Shor, R., Shoham, J. (1986) The enhancing effect of interferon-beta and gamma on the killing of *Leishmania tropica major* in human mononuclear phagocytes *in vitro*. J. Immunol. **136**:3062-3066.

Patterson, R.M., Hill, J.F., Shiel, M.J., and Humphrey, J.D. (1984) Isolation off *Haemophilus somnus* from vaginitis and cervicitis in dairy cattle. Aust. Vet. J. **61**: 301-302.

Payne, N.R., Bellinger,-Kawahara, C.G., and Horwitz, M.A. (1987) Phagocytosis of *Mycobacterium tuberculosis* by human monocytes is mediated by receptors for the third component of complement. Clin. Res. **35**: 617A.

Payne, N.R., and Horwits, M.A. (1987) Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. J. Exp. Med. **166**: 1377-1389.

Pearson,R.D., Harcus, J.L., Symes, P.H., Romito, R. and Donowitz, G.R. (1982) Failure of the phagocytic oxidative response to protect human monocyte-derived macrophages from infection by *Leishmania donovani*. J. Immunol. **129**: 1282-1286.

Pennell, J.R., and Renshaw, H.W. (1977) *Haemophilus somnus* complex: *in vitro* interactions of *Haemophilus somnus*, leucocytes, complement, and antiserums produced from vaccination of cattle with fractions of the organism. Am. J. Vet. Res. **38(6)**: 759-769.

Perlmutter, D.H., Dinarello, C.A., Punsal, P.I., and Coltein, H.R. (1986) Cachectin/tumor necrosis factor regulates hepatic acute phase gene expression. J. Clin. Invest. **78**: 1349-1354.

Pfefferkorn, E.R., Eckel, M., and Rebhun, S. (1986) Interferon gamma suppress the growth of Toxoplasma gondii in human fibroblasts through starvation for tryptophan. Mol. Biochem. Parasitol. **20**: 215-224.

Pfefferkorn, E.R., and Pfefferkorn, L.C. (1981) Toxoplasma gondii: growth in the absence of host cell protein synthesis. Experimental Parasitology **52:** 129-136.

Pfeifer, C.G., (1992) Interaction of *Haemophilus somnus* with bovine leucocytes. M.Sc. Thesis, University of Saskatchewan, Saskatoon, SK.

Pfeifer, C.G., Campos, M., Beskorwayne, T., Babiuk, L.A. and Potter, A.A. (1992) Effect of *Haemophilus somnus* on phagocytosis and hydrogen peroxide production by bovine polymorphonuclear leucocytes. **13:** 191-202.

Philip, R., and Epstein, L.B. (1986) Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon and interleukin-1. Nature (London) **323:** 86-89.

Plant, J., and Glynn, A.A. (1976) Genetics of resistance to infection with *Salmonella typhimurium* in mice. J. Infect. Dis. **133:** 72-78.

Pommier, C.G., Inada, S., Fries, L.F., Takahashi, T., Frank. M.M., Brown, E.J. (1983) Plasma fibronectin enhancers phagocytosis of opsonized particles by human peripheral blood monocytes. J. Exp. Med. 157: 1844-1854.

Portnoy, D.A., Jacks, P.S., and Hinrichs, D.J. (1988) Role of haemolysin for the intracellular growth of *Listeria monocytogenes.* J. Exp. Med. **167:** 1459-1471.

Portoy, D.A., Schreiber, R.D., Cannelly, P., and Tilney, L.G. (1989) γ-Interferon limits access of *Listeria monocytogenes* to the macrophage cytoplasm. J. Exp. Med. **170:** 2141-2146.

Price, R.E., Templeton, J.W., Smith III, R., and Adams, L.G. (1993) Modulation of the intracellular survival of *Brucella abortus* by tuftsin and muramyl dipeptide. Vet. Immunol. Immunopathol. **36:** 265-279.

Qin, Z., Richter, G., Diamanstein, T., and Blankenstein, T. (1989) Structure and evolution of mouse interleukin-6 gene. Mol. Immunol. **26:** 1021-1026.

Quie, P.G., White, J.G., Holmes, B., and Good, R.A., (1967) *In vitro* bactericidal capacity of human polymorphonuclear leucocytes. Diminished activity in chronic granulomatous disease in childhood. J. Clin. Invest. **46:** 668-79.

Ramadori, G., VanDamme, J., Rieder, H., and Meyer, K.-H. zum Buschenfelde. (1988) Interleukin 6, the third mediator of acute phase reaction, modulates hepatic protein synthesis in human and mouse. Comparison with interleukin 1β and tumor necrosis factor-α. Eur. J. Immunol. **18:** 1259.

Raybourne, R.B., and Bunning, V.K. (1994) Bacterium-host cell interactions at the cellular level: Florescent labelling of bacteria and analysis of short-term bacterium-phagocyte interaction by flow cytometry. Infect. Immun. **62(2)**: 665-672.

Reed, S.G., Nathan, C.F., Pipl, D.L., Rodricks, P., Shanebeck, K., Conlon, P.J., and Grabstein, K.H. (1987) Recombinant granulocyte/macrophage colony-stimulating factor activates macrophages to inhibit *Trypanasoma cruzi* and release hydrogen peroxide. Comparison with interferon γ. J. Exp. Med. **166**: 1734-1746.

Reynolds, H.Y., (1985) Respiratory infections may reflect deficiencies in host defence mechanisms. Dis. Mon. **31**: 1-98.

Reynolds, H.Y., Kazmierowski, J.A., and Dale, D.C., (1976) Changes in the composition of canine respiratory cells following irradiation or drug immunosuppression. Proc. Soc. Exp. Biol. Med. **151**: 756-61.

Riesenfeld-Orn, I., Wolpe, S., Garcia-Bustos, J.F., Hoffmann, M.K., and Tuomanen, E. (1989) Production of interleukin-1 but not tumor necrosis factor by human monocytes stimulated with pneumococcal cell surface components. Infect. Immun. **57**: 1890-1893.

Roecklein, J.A., Swartz, R.P., and Yeager, Jr. H. (1992) Non-opsonic uptake of *Mycobacterium avium* complex by human monocytes and alveolar macrophages. J. Lab. Clin. Med. **119**: 772-781.

Romeo, D. Zabucchi, G. and Marzi, T. (1973) Kinetic and enzymatic features of metabolic stimulation of alveolar and peritoneal macrophages challenged with bacteria. Exp. Cell. Res. **78**: 423-432.

Rook, G.A.W., Attrah, R.A., and Foley, N. (1989) The role of cytokines in the immunopathology of tuberculosis, and the regulation of agalactosyl IgG. Lymphokine. Res. **8**: 323-328.

Rook, G.A.W., Steele, M., Ainsworth, M., and Champion, B.R. (1986) Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: comparison of the effects of recombinant γ-interferon on human monocytes and murine peritoneal macrophages. Immunology **59**: 333-338.

Roos, D., Van der Stijl-Neijenhuis. (1980) The oxygen metabolism of human blood monocytes and neutrophils. In: Van Furth R, ed. Mononuclear phagocytes: functional aspects, part II The hague, Netherlands: Martinus Nijhoff, 1219-1241.

Rosen, H. and Gordon, S. and North, R.J. (1989) Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. J. Exp. Med. **170**: 27-37.

Rosenblum, M.G., and Donato, N.J. (1989) Tumor necrosis factor-α: A multifaceted peptide hormone. Crit. Rev. Immunol. **9**: 21-44.

Rosenthal, A.S., and Shevach, E.M. (1973) Function of macrophages in antigen recognition by guinea pig T lymphocytes. 1. requirement for histocompatible macrophages and lymphocytes. J. Exp. Med. **138**: 1194-1212.

Ross, D, and Balm A.J.M. (1980) Oxidative metabolism of monocytes. In: Sbarra AJ, Strauss RR, eds. The reticuloendothelial system A comprehensible treatise: biochemistry and metabolism. New York: Plenum, 189-299.

Ross, G.D., Cain, J.A., and Lachmann, P.J. (1985) Membrane complement receptor type three (CR3) has lectin-like properties analogous to bovine conglutinin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for iC3b. J. Immunol. **134**: 3307-3315.

Ross, G.D., Cain, J.A., Myones, B.L., Newman, S.L., Lachmann, P.J., (1987) Specificity of membrane complement receptor type three (CR3) for β glucans. Complement **4**: 61-74.

Rossi, F. Bellavite, P. and Dobrina, A. et al. (1980) Oxidative metabolism of mononuclear phagocytes. In: Van Furth R. ed. Mononuclear phagocytes: functional aspects, part II The hague, Netherlands: Martinus Nijhoff, 1187-1213.

Rothermel, C.D., Rubin, B.Y., Murray, H.W. (1983) Gamma-interferon is the factor in lymphokine that activates human macrophages to inhibit intracellular *Chlamydia psittaci* replication. J. Immunol. **131**: 2542-2544.

Sample, A.K., and Czuprynski, C.J. (1991) Elimination of hydrogen peroxide by *Haemophilus somnus*, a catalase-negative pathogen of cattle. Infect. Immun. **59(7)**: 2239-2244.

Sandron, D., Reynolds, H.Y., Venet, A., Laval, A.M., Israel-Biet, D., Chretien, J. (1986) Human alveolar macrophage sub-populations isolated on discontinuous albumin gradients: functional data in normal and sarcoid patients. Eur. J. Respir. Dis. **69**: 226-34.

Sansonetti, P.J., Ryter, A., Clerc, P., Maurelli, A.T., and Mounier, J. (1986) Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocyte vacuole and plasmid mediated contact haemolysis. Infect. Immun. **51**: 461-469.

Saunders, J.R., Thiessen, W.A., and Janzen, E.D. (1980) *Haemophilus somnus* infections. I. A ten year (1969-1978) retrospective study of losses in cattle herds in western Canada. Can. Vet. J. **21:** 119-123.

Schlesinger, L., and Horwitz, M.A. (1988) Phagocytosis of leprosy bacilli by human monocytes is mediated by complement receptors CR1 and CR3. Clin. Res. **36:** 582A.

Schlesinger, L.S., and Horwitz, M.A. (1990) Phagocytosis of leprosy bacilli is mediated by complement receptors CR1 and CR3 on human monocyte and complement component C3 in serum. J. Clin. Invest. **85:** 1304-1314.

Schluter, B., Konig, B., Bergmann, U., Muller, F.E., and Konig, W. (1991) Interleukin-6: a potential mediator of lethal sepsis after major thermal trauma-evidence for increased IL-6 production by peripheral blood mononuclear cells. J. Trauma. **31:** 1663-1670.

Schreiber, R.D. (1984) Identification of gamma-interferon as a murine macrophage-activating factor for tumor cytotoxicity. Contemp. Top. Immunobiol. **13:** 171-198.

Schuh, J., and Harland, R. (1991) *Haemophilus somnus* myocarditis versus thrombotic meningoencephalitis in Western Canada. Can. Vet. J. **32:** 439.

Sen, G.C., Lengyel, P. (1992) The interferon system: A birds eye view of its biochemistry. J. Biol. Chem. **267:** 5017-5020.

Shabo, Y., Lotem, J., Rubinstein, M., Revel, M., Clark, S., Wolf, S., Kamon, R., and Sachs, L. (1988) The myeloid blood cell differentiation-inducing protein MG1-2A is interleukin-6 Blood **72:** 2070-2073.

Shapiro, H. (1988) Practical flow cytometry. 2nd edn. New York: Alan R. Liss.

Shellito, J., and Kaltreider, H.B. (1984) Heterogeneity of immunologically function among subfractions of normal alveolar macrophages. Am. Rev. Respir. Dis. **129:** 747-53.

Shigidi, M.A., and Hoerlein, A.B. (1970) Characterization of the *Haemophilus*-like organism of infectious thromboembolic meningoencephalitis of cattle. Am. J. Vet. Res. **31:** 1017-1022.

Sibille, Y., Naegel, G.P., Merrill, W.W., Young, K.R.Jr., Care, S.B., and Reynolds, H.Y. (1987) Neutrophil chemotactic activity produced by normal and activated human bronchoalveolar lavage cells. J. Lab. Clin. Med. **110:** 624-33.

Sibley, L.D., Adams, L.B., and Krahenbuhl, J.L. (1990) Inhibition of interferon-gamma-mediated activation in mouse macrophages treated with lipoarabinomannan. Clin. Exp. Immunol. **80**: 141-148.

Sibley, L.D., Hunter, S.W., Brennan, P.J. and Krahenbuhl, J.L. (1988) Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. Infect. Immun. **56**: 1232-1236.

Sibley, L.D., Krahenbuhl, J.L., Adams, G.M.W. and Weidner, E. (1986) *Toxoplasma* modifies phagosomes by secretion of a vesicular network rich in surface proteins. J. Cell. Biol. **103**: 867-874.

Sibley, L.D., Weidner, E., and Krahenbuhl, J.L. (1985) Phagosome acidification blocked by intracellular *Toxoplasma gondii*. Nature (London) **315**: 416-419.

Silva, S.V.P., and Little, P.B. (1990) The protective effect of vaccination against experimental pneumonia in cattle with *Haemophilus somnus* outer membrane antigens and interference by lypopolysaccharide. Can. J. Vet. Res. **54(3)**: 326-330.

Skamene, E.P., Gros, P., Forget, A., Kongshavn, P.A.L., Charles, C. St., and Yaylor, B.A. (1982) Genetic regulation of resistance to intracellular pathogens. Nature **297**: 506-509.

Smith, H., and Fitzgeorge, R.B. (1964) The chemical basis of the virulence of *Brucella abortus*. V. The basis of intracellular survival and growth in phagocytes. Br. J. Exp. Pathol. **45**: 174-186.

Snyers, L., Fontain, V., and Content, J. (1989) Modulation of interleukin-6 receptors in human cells. Ann. NY Acad. Sci. **557**: 388-393.

Roos, D., Van der Stijl-Neijenhuis. (1980) The oxygen metabolism of human blood monocytes and neutrophils. In: Van Furth R, ed. Mononuclear phagocytes: functional aspects, part II The hague, Netherlands: Martinus Nijhoff, 1219-1241.

Springmeyer, S.C., Altman, L.C., Kopecky, K.J., Deeg, H.J., and Storb, R. (1982) Alveolar macrophage kinetics and function after interruption of canine marrow function. Am. Rev. Respir. Dis. **125**: 347-51.

Stahl, P.D., (1990) The macrophage mannose receptor: current status. Am. J. Respir. Cell. Mol. Biol. **2**: 317-318.

Stephens, L.R., Humphrey, J.D., Little, P.B., and Barnum, D.A. (1983) Morphological, biochemical, antigenic, and cytochemical relationships among *Haemophilus somnus, Histophilus ovis*, and *Actinobacillus seminis*. J. Clin. Microbiol. **17**: 728-737.

Stephens, L.R., and Little, P.B. (1981) Ultrastructure of *Haemophilus somnus*, causative agent of bovine infectious thromboembolic meningoencephalitis. Am. j. Vet. Res. **42:** 1638-1640.

Stephens, L.R., Little, P.B., Wilkie, B.N., and Barnum, D.A., (1981) Infectious thromboembolic meningoencephalitis in cattle: A review. J. Am. Vet. Med. Assoc. **178:** 378-384.

Stephens, L.R., Slee, K.J., Poulton, P., Larcombe, M., and Kosior, E. (1986) Investigation of purulent vaginal discharge in cows, with particular reference to *Haemophilus somnus*. Aust. Vet. J. **63:** 182-185.

Stevens, M.G., Kehril, M.E.Jr., and Canning, P.C. (1991) A colorimetric assay for quantitating bovine neutrophil bactericidal activity. Vet. Immunol. Immunopathol. **28:** 45-56.

Stevens, P., Winston, D.J., Van Dyke, K. (1978) *In vitro* evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: utility in patients with severe neutropenia and cellular deficiency status. Infect. Immun. **22:** 41-51.

Straley, S.C. and Harmon, P.A. (1984) Yersinia pestis grows within phagolysosomes in mouse peritoneal macrophages. Infect. Immun. **45:** 655-659.

Strauss, R.R., Paul, B.B., Jacobs, A.S., and Sbarra, A.J., (1970) Role of the phagocyte in host-parasite interactions: XXII H_2O_2 dependant decarboxylation and deamination by myeloperoxidase and its relationship to antimicrobial activity. J. Reticuloendothel. Soc. **7:** 754-61.

Stuehr, D.J., Gross, S.S., Sakuma, I., Levi, R., and Nathan, C.F. (1989) Activated murine macrophages secrete a metabolite of arginine with the bioactivity of endothelium-derived relaxin factor and the chemical reactivity of nitric oxide. J. Exp. Med. **169:** 1011-1020.

Stuehr, D.J., and Marietta, M.A. (1987) Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-g. J. Immunol. **139:** 518-525.

Stuehr, D.J., and Nathan, C.F. (1989) Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. **169:** 1543-1555.

Sucic, M., Kolevska, T., Kopjar, B. et al., (1989) Accuracy of routine flow-cytometric bitmap selection for three leucocyte populations. Cytometry **10:** 442-447

Suda, T., Yamaguchi, Y., Suda, J., Miura, Y., Okano, A., and Akiyama, Y. (1988) Effect of interleukin-6 (IL-6) on the differentiation and proliferation of murine and human hemopoietic progenitors. Exp. Hematol. **16:** 891-895.

Sweeney, T.D., Castranova, V., Bowman, L. and Miles, P.R. (1981) Factors which affect superoxide anion release from rat alveolar macrophages. Exp. Lung Res. **2:** 85-96.

Tanowitz, H., Wittner, M., Kress, Y., Bloom, B. (1975) Studies of *in vitro* infection by *Trypanasoma cruzi*. I. Ultrastructural studies on the invasion of macrophages and L-cells. Am. J. Trop. Med. Hyg. 25: 25-33.

Tarling, J.D., and Coggle, J.E., (1982) Evidence for the pulmonary origin of alveolar macrophages. Cell Tissue Kinet **15:** 577-84.

Tarr, H.L.A. (1941) Bacteriostatic action of nitrates. Nature (London) **147:** 417-418.

Tauber, A.L. (1987) Protein kinase C and the activation of the human neutrophil NADPH oxidase. Blood **69:** 711-720.

Thompsom, K.G., and Little, P.B. (1981) Effect of *Haemophilus somnus* on bovine endothelial cells in organ culture. Am. J. Vet. Res. **42:** 748-754.

Thomson, M.S., Stringfellow, D.A., and Lauerman, L.H. (1988) Adherence of *Haemophilus somnus* to bovine embryos after *in vitro* exposure. Am. J. Vet. Res. **49(1):** 63-66.

Titus, R.G., Sherry, B., and Cerami, A. (1989) Tumor necrosis factor plays a protective role in experimental murine cutaneous leishmaniasis. J. Exp. Med. **170:** 2097-2104.

Todd, W.J., and Storz, J. (1975) Ultrastructural, cytochemical evidence for the activation of lysosomes in the cytocidal effect of *Chlamydia psittaci*. Infect. Immun. **12:** 638-646.

Tosato, G., and Pike, S.E (1988) Interferon-β2/interleukin 6 is a co-stimulant for human T lymphocytes. J. Immunol. **141:** 1556-1562.

Tosato, G., Seamon, K.B., Goldman, N.D., Sehgal, P.B., May, L.T., Washington, G.C., Jones, K.D., and Pike, S.E. (1988) Monocyte-derived human B-cell growth factor identified as interferon-β2 (BSF-2, IL-6). Science **239:** 502-504.

Tracey, K.J., Beutler, B., Lowry, S.F., Merryweather, J., Wolpe, S., Milsark, I.W., Hariri, R.J., Fahey, T.J., Zentella, A., Albert, J.D., Shires, G.T., and Cerami, A. (1986) Shock and tissue injury induced by recombinant cachectin. Science (Wash. DC) **470:** 234.

Tracey, K.J., Lowry, S.F., Fahey, T.J., Fong, Y., Hesse, D.G., Beutler, B., Manogue, K., Calveno, S.E., Wei, H., Albert, J.D., Cerami, A., and Shires, G.T. (1987) Cachectin/tumor necrosis factor induces lethal septic shock and stress hormone responses in the dog. Surg. Gynecol. Obstet. **164:** 415.

Tracey, K.J., Vlassara, H., and Cerami, A. (1989) Cachectin/tumor necrosis factor. Lancet **1:** 1122-1126.

Tsuyuguchi, I., Kawasumi, H., Takashima, T., Tsuyuguchi, T., and Kishimoto, S. (1990) *Mycobacterium avium-Mycobacterium intracellulare* complex-induced suppression of T-cell proliferation *in vitro* by regulation of monocyte accessory cell activity. Infect. Immun. **58:** 1369-1378.

Unanue, E.R., and Allen, P.M. (1987) The basis for the immunoregulatory role of macrophages and other cells. Science. **236:** 551-557.

Van Dilla, R., Langlois, G., Pinkel, D., Yauko, D., and Hadley, W.K. (1983) Bacterial characterization by floe cytometry. Science **220:** 620-622.

van Dissel, J.T., Stikkelbroeck, J.J.M., Michel, B.C., van den Barselaar, M.T., Leijh, P.C.J., and van Furth, R. (1987) Inability of recombinant interferon-γ to activate the antibacterial activity of mouse peritoneal macrophages against *Listeria monocytogenes* and *Salmonella typhimurium*. J. Immunol. **139:** 1673-1678.

van Dreumel, A.A., Curtis, R.A., and Ruhnke, H.L. (1970) Infectious thromboembolic meningoencephalitis in Ontario feedlot cattle. Can. Vet. J. **11:** 125-130.

Van Snick, J. (1990) Interleukin-6, an overview. Annu. Rev. Immunol. **8:** 253-278.

Van Snick, J., Vink, A., Cayphas, S., and Uyherhove, C. (1987) Interleukin-HP 1, a T cell-derived hybridoma growth factor that supports the *in vitro* growth of murine plasmacytomas. J. Exp. Med. **165:** 641-649.

Varesio, L., Cox G.W., Pulkki, K., Musso, T., Latham, P.S., Gusella, G.L., and Espinoza-Delgado,, I. (1993) Activation of macrophages and monocytes by interferon gamma and interleukin-2, in Klostergaard J. Lopez-Berestein G. (eds): Mononuclear phagocytes in cell biology. Boca Raton, FL, CRC, p 119.

Vincendeau, P., and Daulouede, S. (1991) Macrophage cytotoxic effect on *Trypanosoma musculi* involves an L-arginine-dependent mechanism. J. Immunol. **146:** 4338-4343.

Von Luginbuhl, A., Kupfer, U. (1980) Bakteriologische befunde im geschlechtsapparat von kuhen im puerperium. Mitteilung II. Schweiz Arch Tierheilkd **122:** 695-705.

Waage, A., Brandkaeg, P., Halstensen, A., Kierulf, P., and Esperik, T. (1989) The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin-6, interleukin-1, and fatal outcome. J. Exp. Med. **169**: 333-338.

Waldham, D.D., Hall, R.F., Meinershagen, W.A., Card, C.S., and Frank, F.W. (1974) *Haemophilus somnus* infection in the cow as a possible contributing factor to weak calf syndrome: Isolation and animal inoculation studies. Am. J. Vet. Res. **35**: 1401-1403.

Walker, R.L., Jr.(1985) Studies on *Histophilus ovis* associated epidydimitis in rams. Ph.D. thesis. University of California, Davis, CA, pp. 232.

Walker, R.L., Biberstein,, E.L., Pritchett, R.F., and Kirkham, C. (1985) Deoxyribonucleic acid relatedness among *"Haemophilus somnus"* , *"Haemophilus agni","Histophilus ovis",Actinobacillus seminis"*, and *Haemophilus influenzae*. Internat. J. Syst. Bacteriol. **35(1)**: 46-49.

Ward, P.A.,Duque, R.E., Sulavik, M.C. and Johnson, K.J. (1983) *In vitro* and *in vivo* stimulation of rat neutrophils and alveolar macrophages by immune complexes. Am. J. Pathol. **110**: 297-309.

Ward, G.E., Nivard, J.R., and Maheswaran, S.K. (1984) Morphological features, structure, and adherence to bovine turbinate cell of three *Haemophilus somnus* variants. Am. J. Vet. Res. **45**: 336-338.

Weide, K.D., Hibbs, C.M., and Anthony, H.D. (1964) Diagnosis of bovine encephalitides. 68th Annu Meet US Livestock Sanit Assoc

Weiser, W.Y., Van Niel, A., Clark, S.C., David, J.D., and Remold, H.G. (1987) Recombinant human granulocyte/macrophage colony-stimulating factor activates intracellular killing of *Leishmania donovani* by human monocyte-derived macrophages. J. Exp. Med. **166**: 1436-1446.

Welch, W.D. (1980) Correlation between measurements of the luminol-dependant chemiluminescence response and bacterial susceptibility to phagocytosis. Infect. Immun. **30**: 370-374.

Welsh, R. (1981) Natural cell-mediated immunity during viral infections. Curr. Topics. Microbiol. Immunol. **92**: 83-106.

Widders, P.R., Dorrance, L.A., Yarnall, M., and Corbeil, L.B. (1989a) Immunoglobulin binding activity among pathogenic and carrier isolates of *Haemophilus somnus*. Infect. Immun. **52(7)**: 639-642.

Widders, P.R., Dowling, S.C., Gogolewski, R.P., Smith, J.W., and Corbeil, L.B. (1989b) Isotypic antibody response in cattle infected with *Haemophilus somnus*. Rev. Vet. Sci. **46(2)**: 212-217.

Widders, P.R., Paisley, L.G., Gogolewski, R.P., Evermann, J.F., Smith, J.W., and Corbeil, L.B. (1986) Experimental abortion and the systemic immune response to *"Haemophilus somnus"* in cattle. Infect. Immun. **54(2)**: 555-560.

Williams, D.M., Magee, D.M., Bonewald, L.F., Smith, J.G., Bleicker, C.A., Byrne, G.I., and Schacter, J. (1990) A role *in vivo* for tumor necrosis factor alpha in host defence against *Chlamydia trachomatis*. Infect. Immun. **58**: 1572-1576.

Williams, J.M., Smith, G.L., and Nurdock, F.M. (1978) Immunogenicity of *Haemophilus somnus* bacterin in cattle. Am. J. Vet. Res. **39**: 1756-1762.

Wilson, J.G., Andriopoulos, N.A., and Fearon, D.T. (1987) CR1 and cell membrane proteins that bind C3 and C4. A basic and clinical review. Immunol. Res. **6**: 192-209.

Wilson, M.E., and Pearson, R.D. (1988) Roles of CR3 and mannose receptors in the attachment and ingestion of *Leishmania donovani* by human mononuclear phagocytes. Infect. Immun. **56**: 363-369.

Wilson, M.E., Trush, M.A., Van Dyke K. et al. (1978) Luminol-dependent chemiluminescence analysis of opsonophagocytic dysfunctions. J. Immunol. Methods **23**: 315-326.

Wilson, C.B., Tsai, V., and Remington, J.S. (1980) Failure to trigger the oxidative burst by normal macrophages. Possible mechanism for survival of intracellular pathogens. J. Exp. Med. **151**: 328-346.

Wilson, C.B., and Westfall, J. (1985) Activation of neonatal and adult human macrophages by alpha, beta, and gamma interferons. Infect. Immun. **49**: 351-356.

Wirth, J.J., and Kierszenbaum, F. (1984) Fibronectin enhances macrophage association with invasive forms of *Trypanosoma cruzi*. J. Immunol. **133**: 460-464.

Wolf, J.E., Abegg, A.L., Travis, S.J., Kobayashi, G.S., Little, J.R. (1989) Effect of *Histoplasma capsulatum* on murine macrophage functions: inhibition of macrophage priming, oxidative burst, and antifungal activities. Infect. Immun. **57**: 513-519.

Wolf, J.E., Kerchberger, V., Kobayashi, G.S., and Little, J.R. (1987) Modulation of the macrophage oxidative burst by *Histoplasma capsulatum*. J. Immunol. **138**: 582-586.

Wong, G., Witek-Giannotti, J., Temple, P., Kriz, R., Ferenz, C., Hewick, R., Clark, S., Ikebuchi, K., and Ogawa, M. (1988) Stimulation of murine haemopoitic colony formation by human IL-6. J. Immunol. **140:** 3040-3044.

Wright, S.D., Licht. M.R., Craigmyle, L.S., and Silverstein, S.C. (1984) Communication between receptor for different ligands on a single cell: ligation of fibronectin receptors induces a reversible alteration in the function of complement receptors on cultured human monocytes. J. Cell. Biol. **99:** 336-339.

Wright, S.D., and Silverstein, S.C. (1983) Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. J. Exp. Med. **158:** 2016-2023.

Wyler, D.J., Sypek, J.P., McDonald, J.A. (1985) *In vitro* parasite-monocyte interactions in human *Leishmania:* Possible role of fibronectin in parasite attachment. Infect. Immun. **49:** 305-311.

Wyrick, P.B., and Brownridge, E.A. (1978) Growth of *Chlamydia psittaci* in macrophages. Infect. Immun. **19:** 1054-1060.

Yachie, A., Takano, N., Ohta, K., Uehara, T., Fujita, S., Miyawaki, T., and Taniguchi, N. (1992) Defective production of interleukin-6 in very small premature infants in response to bacterial pathogens. Infect. Immun. **60:** 749-753.

Yachie, A., Takano, N., Yokoi, T., Kato, K., Kasahara, Y., Miyawaki, T., and Taniguchi, N. (1990) The capability of neonatal leucocytes to produce IL-6 on stimulation assessed by whole blood culture. Pediatr. Res. **27:** 227-233.

Yamamoto,K., and Johnston, Jr. R.B. (1984) Dissociation of phagocytosis from stimulation of the oxidative metabolic burst in macrophages. J. Exp. Med. **159:** 405-416.

Yarnall, M., and Corbeil, L.B. (1989) Antibody response to *Haemophilus somnus* Fc receptor. J. Clin. Microbiol. **27(1):** 111-117.

Yarnall, M., Gogolewski, R.P., and Corbeil, L.B. (1988a) Characterization of two *Haemophilus somnus* Fc receptors. J. Gen. Microbiol. **134:** 1993-1999.

Yarnall, M., Widders, P.R., and Corbeil, L.B. (1988b) Isolation and characterization of Fc receptors from *Haemophilus somnus.* Scand. Immunol. **28:** 129-137.

Yu, R., Gray-Owen, S.D., Ogunnariwo, J., and Schryvers, A.B. (1992) Interaction of ruminant transferrin with transferrin receptors in bovine isolates of *Pasteurella haemolytica* and *Haemophilus somnus.* Infect. Immun. **60(7):** 2992-2994.

Zhan, Y.F., Stanley, E.R., and Cheers, C. (1991) Prophylaxis or treatment of experimental brucellosis with interleukin-1. Infect. Immun. **59**: 1790-1794.

Zhang, L., Goren, M.B., Holzer, T.J., Andersen, B.R. (1988) Effect of *Mycobacterium tuberculosis*-derived sulfolipid-I on human phagocytic cells. Infect. Immun. **56**: 2876-2883.