Interaction of *Moraxella bovis* with bovine neutrophils: Identification and partial characterization of a leukocidic effect

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PREFACE

This thesis was prepared using the alternate format. It consists of one paper prepared in the format of the American Journal of Veterinary Research and accepted for publication by this journal. A general introduction is followed by a review of the literature and the accepted paper. A general discussion, literature cited, and acknowledgements conclude the thesis. The master's candidate, Patricia Hoien-Dalen, is the senior author of the paper and principal investigator of the study.

GENERAL INTRODUCTION

Infectious bovine keratoconjunctivitis (IBK) is economically the most important ocular disease of cattle. The high morbidity rate, which can reach 80% in an infected herd,[1] results in reduced liveweight gain and milk production.[1,2] Lost revenue from decreased production, combined with the high cost of treatment, produces a severe economic impact on cattle producers in the United States. The latest available information conservatively estimates the cost of IBK to the U.S. cattle industry to be in excess of 200 million dollars annually.[2]

Moraxella bovis is the etiologic agent of IBK.[3,4,5,6,7] Although other factors such as ultraviolet light,[8,9,10] *Mycoplasma bovoculi*,[6,10,11,12,13] and face flies[14,15,16,17] have been demonstrated to contribute to the establishment or dissemination of IBK, *M. bovis* is the only organism for which Koch's postulates have been fulfilled.[3,18,19,20,21,22,23,24,25,26,27]

The initial clinical signs of IBK are consistent with anterior segment irritation and ocular discomfort.[7] These signs include blepharospasm,[1,7] photophobia[1,7] and a serous discharge[1,7,28] which soon becomes purulent.[1,7] The progression and severity of clinical disease is variable;[29] the initial signs may be followed by corneal edema,[1,29] opacity,[1,28] and ulceration,[1,7,29] with less severely affected eyes becoming vascularized and healing rapidly.[7] Less commonly, keratoconus develops,[29] the corneal ulcer progresses to a

descemetocoele,[1,29] and penetration of the anterior chamber occurs.[1,29] Microscopically, large numbers of neutrophils have been observed at various stages and in various areas of the cornea during *Moraxella bovis* infection.[27,30,31,32] Although several investigators have suggested a role for neutrophils in the pathogenesis of IBK,[27,30,31,32] characterization of the direct interaction of *Moraxella bovis* with neutrophils has received little attention.

This study was undertaken to provide information concerning the interaction of *Moraxella bovis* and bovine neutrophils. The objectives were i) to determine if *Moraxella bovis* is capable of producing a detrimental effect(s) on bovine neutrophils, ii) to characterize this effect(s) and, iii) to compare this effect(s) with recognized cytotoxins of *M. bovis*, primarily the *M. bovis* hemolysin.

LITERATURE REVIEW

Virulence Factors

Fimbriae

Surface structures, called fimbriae or pili, are associated with the ability of gram negative bacteria to adhere to epithelial cells.[33] Adherence plays an important role in a bacterium's ability to compete with commensal microorganisms and to successfully colonize host tissues.[34,35] Fimbriae allow attachment of bacteria to various surfaces and prevent removal by both specific and nonspecific host defenses; resulting in bacterial proliferation and colonization.[35] For example, the pili of *Neisseria gonorrhoeae* are believed to prevent phagocytosis by leukocytes.[36,37,38] Contribution of adherent microorganisms to the development of pathological lesions and clinical disease may result from the adsorption of secreted bacterial toxins by the host or physical disruption of normal cellular function by adherent bacteria.[35]

Moraxella bovis is a fimbriated organism,[39,40,41] with the fimbriae originating from small opacities in the outer wall of the bacterium.[41] They are fairly straight, single stranded, unbranched, and have a peritrichous distribution.[41] A single strain of *M. bovis* is capable of producing more than one type of pilin protein and strain Epp63 produces two such pilins; alpha and beta.[42] Alpha and beta pilin

share approximately 70% overall DNA sequence homology and arise from a single gene locus by a process of chromosomal rearrangement.[43,44] Analysis of other M. bovis strains indicates that the ability to produce these two types of pilin may be characteristic of the species.[44]

The involvement of fimbriae in the initial colonization of ocular surfaces by M. bovis is well documented.[39,44,45] Conjunctival infection experiments conducted in calves, utilizing fimbriated and nonfimbriated variants of M. bovis, revealed that only fimbriated variants were able to colonize the conjunctival mucosa.[39] Fimbriated M. bovis strain 118F was shown to adhere significantly better to ocular surfaces than strain 118F/4-2, a nonfimbriated variant.[45] Ocular inoculation of calves with beta-piliated M. bovis Epp63 resulted in a significantly higher rate of colonization than did inoculation with either alpha-piliated or nonpiliated strain Epp63 organisms.[44]

In addition to enhanced colonization of ocular surfaces attributable to the presence of fimbriae, a direct correlation exists between the presence of fimbriae and the ability of M. bovis to produce clinical disease. Pathogenic stains of M. bovis adhered in vitro and were demonstrated to be piliated by transmission electron microscopy.[46] In contrast, nonpathogenic strains did not adhere in vitro and were nonpiliated. Suspensions of nonadherent isolates, or adherent strains whose adhesins were chemically disrupted, were shown to be nonpathogenic.[47] Instillation of suspensions, containing either

adherent isolates or adherent isolates treated with $MgCl_2$ to disrupt the fimbriae,[48] into different conjuctival sacs of the same animal revealed that only nontreated, adherent suspensions produced IBK.[47] Both beta-piliated and alpha-piliated variants of strain Epp63 were significantly more pathogenic, as judged by the production of ocular lesions, than were nonpiliated variants.[44] No significant difference was observed between the pathogenicity of alpha and beta-piliated variants once infection was established, indicating that beta piliation is associated with enhanced colonization but not with enhanced pathogenicity once infection is established.[44]

Hemolysin

Although fimbriae play an important role in the establishment of *M. bovis* in the eye, the presence of fimbriae alone may not be sufficient for virulence of the organism.[39,44] Nonhemolytic *M. bovis* can become established in the eye in large numbers but does not produce signs of IBK under field conditions.[22] In contrast, hemolytic and fimbriated *M. bovis* strains are virulent and generally can be isolated from acute cases of IBK.[10,22,23,49] Defining the role of hemolysin in the production of clinical IBK has been the subject of several studies. All calves, except one, experimentally infected with a hemolytic strain of *M. bovis* developed IBK.[27] Subsequent culture of the unaffected calf recovered a majority of nonhemolytic organisms; demonstrating that the loss of hemolytic ability resulted in the loss of virulence. Findings

of a study conducted by Pugh and Hughes[22] further support the correlation between hemolysin production and the occurrence of clinical IBK. These findings included; nonhemolytic strains did not produce keratitis, eyes that did not develop IBK with nonhemolytic strains developed IBK when subsequently exposed to hemolytic *M. bovis*, and nonhemolytic strains which did not produce IBK did produce hemolytic variants which caused IBK.

Based upon experimental evidence, investigators have concluded that the *M. bovis* hemolysin is significantly involved in the production of mortality in experimentally infected animals.[50,51,52] Hemolytic strains of *M. bovis* produced mortality when inoculated into chicken embryos and intraperitoneally into mice.[51] Two studies demonstrated a significantly greater mortality rate in mice inoculated intraperitoneally with hemolytic strains of *M. bovis* compared to mice inoculated with nonhemolytic strains.[50,52] In one study, greater than 88% of those inoculated with live hemolytic *M. bovis* died within 1-3 hours, whereas the nonhemolytic strains produced 25% mortality in 5-15 hours.[50] In the second study, nonhemolytic mutants, differing from their hemolytic parental strains only in their ability to produce hemolysin, killed 5/55 mice within six hours after intraperitoneal injection.[52] In contrast, death of 48/56 mice inoculated with hemolytic strains occurred within the same time period.

The hemolysin of *M. bovis* is produced during logarithmic phase growth[53,54,55] and is a filterable product of bacterial cells.[54,55] It

is destroyed by heat, formalin and trypsin;[53,54,55] characteristics which suggest the toxin is protein in nature. The hemolytic activity has been described as being enhanced[54,55] or dependent[53] on the presence of calcium. Magnesium was described as being capable of substituting for calcium in some studies.[54,55] The hemolysin was found to be very labile to routine procedures of concentration,[55] purification,[55] and storage.[54] Although this instability may be due to production of extracellular proteolytic enzymes by *M. bovis*,[55] addition of the protease inhibitors diethylpyrocarbonate and trypsin soybean inhibitor did not prevent loss of hemolytic activity.[54]

Additional virulence factors

While fimbriation and hemolysin production are factors which have been demonstrated to be directly involved in disease production, other properties of *M. bovis* have a less well defined role in pathogenesis. *M. bovis* is proteolytic[56,57,58] with demonstrated fibrinolytic,[56,59] gelatinase,[56,57,58,61] and caseinase[56,61] activity. Lipases have also been shown to be produced by *M. bovis*.[56,60] The enzymatic activity of *M. bovis* may contribute to IBK lesions by the production of localized tissue damage.[56]

The toxic properties of whole *M. bovis* organisms and various cellular fractions have been investigated. The high mortality produced when viable *M. bovis* cultures were injected by various routes into mice, rabbits, and chicken embryos was considered to be consistent with

the presence of one or more toxins.[51] Toxins have been suggested to account for the severe ocular lesions of IBK[50,62,63] and Pugh et al.[64] concluded that virulent M. bovis probably produces several toxins including endotoxins, exotoxins, and possibly specific oculopathic substances.

A dermonecrotic toxin, producing localized skin lesions following injection, has been demonstrated to be present in the cell wall fraction of M. bovis.[50,62,64] Cell extracts of M. bovis induced a "shock syndrome", as well as hemorrhage, inflammation and congestion of various organs when inoculated into cattle, mice, and guinea pigs.[64] Conjunctivitis and other ocular lesions were also produced by these extracts.[64] The absence of heptose, a nearly universal component of bacterial endotoxin,[65,66] the proteinacious nature, and the heat-lability of the toxic effect lead to the conclusion that an exotoxin was likely responsible for the cell extracts effects.[64] M. bovis culture filtrates have been demonstrated to produce lethal effects in rabbits,[63] further substantiating the presence of an exotoxin(s).

Recent investigations have examined the toxic effects of M. bovis on cells of the immune system. Live whole cell preparations of M. bovis isolates, which readily induced IBK in vivo, were able to kill high numbers of bovine monocytes and goat macrophages in vitro.[67] Crude cell wall preparations were only a third as toxic as whole cell preparations and heat-killed bacteria were completely nontoxic. Live M. bovis organisms, at a ratio of ten bacteria per neutrophil, produced

a rapid cytotoxic effect on cultured bovine neutrophils.[68] Cytoplasmic vacuolation and cellular swelling were observed within five minutes of incubation by light microscopy and scanning and transmission electron microscopy. Additional changes observed by electron microscopy included pitting of the cell surface, loss of microplicae, rounding of affected cells, and extensive neutrophil degranuation. All neutrophils were lysed by thirty minutes incubation. The cytotoxicity of whole M. bovis organisms and broth culture filtrates was further characterized using ^{51}Cr labeled bovine and human neutrophils.[69] Hemolytic M. bovis isolates were toxic for bovine neutrophils and the toxic activity was dependent on the presence of calcium. Toxicity of whole M. bovis organisms was eliminated by treatment with formalin and sodium azide. Sterile filtrates of log phase cultures were also toxic for bovine neutrophils and toxicity was eliminated by heat and trypsin. None of the M. bovis isolates tested were cytotoxic for human neutrophils and bacterial homogenates, filtrates of stationary phase cultures, and nonhemolytic isolates were noncytotoxic for bovine neutrophils. Kagonyera et al.[69] concluded that the nontoxic nature of nonhemolytic isolates, the heat lability, and the calcium dependency of both hemolytic and cytotoxic activity indicated that the two toxins were related.

Live M. bovis organisms and log phase culture filtrates were demonstrated to be cytotoxic for cultured bovine corneal epithelial cells.[68] Corneal cell changes produced by filtrates were similar to

but less extensive than those produced by live bacteria. Extensive disruption of the monolayer, including areas of complete cell necrosis and sloughing, was observed by light microscopy after ten minutes incubation of live M. bovis with corneal cells. Scanning electron microscopy revealed extensive pitting of corneal cell membranes after ten minutes incubation. By thirty minutes incubation, large areas of the monolayer were devoid of epithelial cells and most of the cells were lysed. Kagonyera et al.[68] concluded that toxin-mediated lysis of neutrophils and corneal epithelial cells may play a significant role in the pathogenesis of IBK.

Pit-like depressions are produced by the adherence of M. bovis organisms to the dark cells of the bovine cornea.[27,32,70,46] The production of these pits on the corneal surface may be the result of bacterial digestion,[70] mechanical factors,[70] or the action of an extracellular product of M. bovis.[46,68] The relationship between the pitting factor, extracellular toxins including the M. bovis hemolysin, and the numerous enzymes of M. bovis has not been established. The pitting factor may assist in penetration of the cornea[46] and play a role in the early stages of infection.[70,46]

Neutrophils in Corneal Lesions

Contribution of neutrophils to corneal lesions

The transparency of the healthy cornea is it's major attribute and results from a combination of several factors. These factors include a regular, nonkeratinized, and nonpigmented surface epithelium,[29] a cell-poor, avascular stroma composed of very thin collagen fibrils arranged in orderly lamellae,[29,71] and a high degree of stromal dehydration.[29]

Loss of transparency is the most obvious indication of corneal disease and may result from disruption of the orderly array of collagen fibrils.[71] Opacity may also result from corneal injury, which allows rapid uptake of lacrimal water through damaged corneal epithelium or incompetent endothelium and leads to stromal edema.[29,72] Healing of corneal wounds occurs by sliding together of the epithelial edges to cover the denuded area[29,72] followed by mitosis.[29] Deeper wounds heal by epithelial sliding, mitosis and stromal fibroplasia.[29]

Neutrophils are attracted to the injured cornea by inflammatory mediators, including proteases released by injured epithelium,[29] the synthetic chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP),[73] and the fifth component of complement (C5).[73] These inflammatory cells reach the cornea within a few hours after injury via the tear film and limbic venules.[29] Once in the cornea, neutrophils perform various beneficial functions, including degradation of damaged stromal collagen, stimulation of fibroplasia and vascularization, and

control of bacterial proliferation.[29] However, the presence of neutrophils in the cornea is not always beneficial to the host. An uncontrolled inflammatory response within the limited confines of the eye may severely damage those tissues on which vision critically depends.

Experiments utilizing guinea pigs made neutropenic by total body irradiation illustrate the role of neutrophils in the development of corneal lesions following inoculation with Pseudomonas aeruginosa.[74,75,76] Neutropenic guinea pigs had fewer neutrophils present[74,75] and significantly more viable Pseudomonas aeruginosa organisms[74] in the corneal stroma than did nonneutropenic animals. However, the neutropenic guinea pigs had significantly less corneal edema[75] and less severe corneal damage.[74] The increase in corneal edema[75] and clinical evidence of infection[76] were directly proportional to the corneal ingress of radiolabeled polymorphonuclear leukocytes (PMNs).

PMNs infiltrated the corneal stroma and produced ulceration in the eyes of rabbits with alkali induced corneal damage.[77] However, application of methylmethacrylate lens, which prevented the infiltration of PMNs, prevented the development of ulcers in other alkali burned corneas in the study. Highly active PMNs, believed to be involved in degradation of the stromal matrix, were observed in the ulcerated corneas by phase-contrast microscopy. In addition to direct involvement in the production of corneal ulcers, neutrophils are also

involved in delayed healing of wounds produced by other means. Both intact PMNs and PMN lysates produced significant retardation of wound healing after addition to abraded rat corneas.[78]

Even without predisposing factors such as bacteria and previous corneal injury, the addition of peripheral bovine leukocytes to the epithelial[79] or endothelial[80] surface of isolated corneas results in significant tissue damage. Leukocytes produced ulceration and destruction of the full thickness of the epithelial layer[79] and endothelial cell changes included swelling, membrane thickening, cytoplasmic vacuolization, and changes in nuclear shape.[80] The endothelial cells were demonstrated to be nonviable by trypan blue dye exclusion.[80]

Contribution of lysosomal enzymes to corneal lesions

Lysosomal enzymes are believed to be the component of neutrophils which contribute to ocular lesion development. PMN lysates contained more consistent levels of cellular enzymes and produced more consistent and greater delays in epithelial wound healing than did whole PMNs.[78] Of the many lysosomal enzymes contained in neutrophils, those capable of degrading stromal collagen would be expected to be of primary importance in the development of corneal lesions since 80% of the solid matter of the cornea is composed of collagen.[72]

Neutrophils have, in fact, been shown to contain collagenases and latent and active collagenases have been purifed from human

polymorphonuclear leukocytes using a variety of methods.[81,82,83] These collagenases have the specificity of true mammalian collagenases[82,83] and were found to be localized in the specific granules of PMNs by zonal sedimentation.[83] Release of collagenase from these granules is believed to occur when neutrophils either engage in phagocytosis or are subjected to other stimuli.[83] Experimentally, activation of latent PMN collagenase could be regulated by variation in the redox potential of the coupled glutathione cycle,[82,84] leading to the conclusion that activation of the released collagenases in vivo may be linked to the phagocytosis-associated respiratory burst.[84]

Phagocytosis not only releases collagenases from granular stores[83] and activates latent collagenases,[82,84] but extracellular release of lysosomal enzymes (including collagenases) may occur during phagocytosis.[85,86] This extracellular release occurs when the phagocytic vacuole is open to the extracellular space for a period of time.[85,86] In such a situation, collagenases could be liberated into an area of inflammation and contribute to further localized tissue damage.[82]

PMNs are believed to be the source of stromal collagenase in the ulcerating cornea.[87,88] Collagenase and proteolytic enzymes capable of degrading proteoglycans were found in ulcerated corneas after the injection of small numbers of heat-inactivated _Pseudomonas aeruginosa_.[89] The high correlation between the enzyme levels and extent of PMN infiltration indicated that the PMNs were the most likely

source of these enzymes.

Grossly fragmented collagen fibrils, similar to those observed after bacterial collagenase injections, were observed by Rowsey et al.[90] on electron microscopic evaluation of rabbit corneas injected with concentrated PMN lysosomal preparations. Van Horn et al.[91] observed degranulating neutrophils, attracted to the corneal stroma by the intracorneal injection of a small number of viable P. aeruginosa organisms, closely associated with necrotic cells and collagen breakdown products in the stroma. The ultrastructural changes observed[91] were remarkably similar to those described by Rowsey et al.[90]

Neutrophils in IBK Lesions

Presence of neutrophils in IBK lesions

The presence of neutrophils in the ocular lesions of IBK is well documented. Neutrophils have been observed on the corneal surface,[27,32] between the ulcerated epithelium and adjacent non-ulcerated epithelium,[31,32] in the corneal stroma,[27,30,31] and within the endothelial layer.[27] Neutrophils within the endothelial layer were adhered to endothelial cells, some of which had undergone cytolysis.[27]

Severe ulceration was observed 24, 45 and 72 hours after inoculation of M. bovis into the eyes of gnotobiotic calves.[27] Using scanning electron microscopy, fibrin, cellular debris, mucus, and

leukocytes were seen to fill the corneal ulcers. Swollen or necrotic epithelial cells were common in areas of neutrophilic infiltration.[27] Neutrophils have also been observed in more chronic IBK lesions.[31] Eight days after M. bovis inoculation, light microscopy lesions common to dexamethasone-treated, hydroxyurea-treated, and control calves included corneal epithelial cell necrosis and sloughing. Transmission electron microscopy demonstrated necrotic epithelial cells separated by large intercellular gaps and degenerative inflammatory cells.[31]

Large numbers of neutrophils have been observed to infiltrate the corneal stroma underlying ulcers in naturally infected field case calves,[30] experimentally infected conventional calves,[30,31] and experimentally infected gnotobiotic calves.[27,30] Stromas were characterized by edema and swollen fibroblasts.[27] The pattern of stromal lamellae was disrupted,[27,30,31] some lamellae were lost,[31] and in some cases neutrophils were in linear arrays, resembling the original collagen layers.[30] Viable and degenerate bacteria were observed in phagosomes of neutrophils,[27,30] and neutrophils in the corneal stroma were in various stages of degranulation and degeneration.[27,30] Stromal lesions, closely resembling those in cattle, have been produced by the intracorneal injection of M. bovis into mice.[92] Neutrophils infiltrated the stroma and were seen, along with M. bovis organisms, associated with disorganized collagen fibers. Viable appearing bacteria and bacterial debris were seen within the cytoplasm of neutrophils in the lesions.[92]

Contribution of neutrophils to IBK lesions

The combination of one or more invasive factors produced by *M. bovis* and the acute inflammatory response of the host to this invasion may be responsible for the pathogenesis of corneal lesions produced by *M. bovis*.[27] While early IBK lesions may largely be the result of bacterial factors and invasiveness,[31] later lesions may be due to an interaction between the bacterium and the host's inflammatory cells.[27,31] A significant role for inflammatory cells in the pathogenesis of IBK is supported by numerous observations of neutrophils in severe IBK lesions without the concurrent presence of *M. bovis* organisms.[27,30,31,32]

No specific feature, other than the infiltration of neutrophils, was seen to account for the opacity surrounding corneal ulcers,[30] nor was *M. bovis* found on the ulcerated surface or the adjacent damaged epithelium[32] after infection with *M. bovis*.[30,32] Degenerative neutrophils were the only infiltrate in some areas of stromal fibril disruption[31] and electron-dense granules observed in the stroma in the later stages of disease were believed to be neutrophil-mediated breakdown products of collagen, proteoglycan, or both.[27] Severe corneal lesions, which included edema, enlargement of ulcers, and neovascularization, were present in 16/16 calves eight days after inoculation with *M. bovis*.[31] However, only one of these corneas was found to contain bacteria at this stage of the disease. These observations have led to the conclusion that the enlargement of corneal

ulcers late in the disease process is due to the influx of inflammatory cells[31,32] and to destruction of the collagen matrix by neutrophil-produced enzymes,[27,32] rather than to massive intracorneal growth of *M. bovis*.[31]

Production of Leukotoxic and Hemolytic Substances by Gram Negative Bacteria

Escherichia coli alpha-hemolysin

Several species of gram negative bacteria produce leukotoxic substances which are considered to be virulence factors of these organisms. The *Escherichia coli* alpha-hemolysin, which possesses both leukotoxic and hemolytic activity,[93,94,95,96] is perhaps the best characterized of these toxins. The importance of alpha-hemolysin as a virulence factor is demonstrated by the disproportionately high percentage of extraintestinal *E. coli* isolates which produce hemolysin.[97,98,99,100,101,102] For example, Minshew et al.[100] found that only 5% of the *E. coli* organisms in the normal fecal flora of humans were hemolytic, while 35-59% of the *E. coli* isolates causing extraintestinal infections such as bacteremia, septicemia, and urinary tract infections were hemolytic. A study of the relationship between hemolytic isolates of *E. coli* and the incidence of urinary tract infection found that 54% of the isolates from urinary tract infections were hemolytic while only 17% of those from the feces of normal

individuals produced hemolysin.[98] Survival of invading hemolytic *E. coli* would be enhanced by destruction of leukocytes,[93] demonstrating a role for hemolysin production in initiating or sustaining extraintestinal infections.[100]

Alpha-hemolysin is produced during the logarithmic phase of growth[95,96,103,104,105,106,107,108] and production declines as cells enter the stationary phase.[103,105,106,108] The *E. coli* alpha-hemolysin produces a rapid cytotoxic effect on host cells[96] which is both dose and time dependent.[93,96] The leukotoxic activity is neutralized by specific antisera, heat, and proteases.[93] Further evidence for the proteinacious nature of the *E. coli* alpha-hemolysin includes the demonstrated destruction of hemolytic and leukotoxic activities by trypsin[93,107,109] and chymotrypsin,[107] and the association of hemolytic activity with major protein peaks during purification.[97] Alpha-hemolysin is present in cell-free supernatants of *E. coli* cultures[94,95,96,102,103,105,108] and is filterable.[93,106,110]

Cytotoxicity is believed to be initiated by the local effect of alpha-hemolysin on the plasma membrane of red blood cells (RBCs)[111,112] and leukocytes.[96] The necessity for close approximation of the toxin and the plasma membrane explains why the effect is seen primarily on phagocytes which attempt to attach and ingest the microorganisms.[96] Although the exact role calcium plays in the interaction between alpha-hemolysin and host cells is unknown, Snyder and Zwadyk[107] demonstrated that calcium or strontium, but not barium or magnesium, was required

for activity. This calcium requirement has been confirmed by others.[109,113]

Calcium may be required for formation of a hemolysin-RBC complex, for maintaining this complex during the prelytic phase, or for both of these activites.[109,113] Calcium has been observed by electron microscopy to cause alpha-hemolysin to change from elongated amorphous aggregates to regular spherical structures.[113] This structural change may result in activation of the toxin. It has also been proposed that a single alpha-hemolysin molecule may bind to an RBC and acting as a calcium ionophore, create a membrane channel.[111,112] The resulting influx of ions, especially calcium, could result in the triggering of intracellular reactions[111] or cause swelling and lysis due to the concomitant influx of water.[112] Bhakdi et al.[114] induced a rapid efflux of cellular K^+ and influx of $^{45}Ca^{2+}$, as well as influx of $[^{14}C]$mannitol and $[^{3}H]$sucrose, by treating erythrocytes suspended in dextran-containing buffer with _E. coli_ alpha-hemolysin. It was concluded that _E. coli_ hemolysin may partially insert into the lipid bilayer of cell membranes and damage these membranes by the generation of discrete, hydrophilic transmembrane pores with an effective diameter of approximately 3 nm.[114] In contrast to the findings of Jorgensen et al.,[111,112] the toxin pore was felt to be nonspecific in nature due to the influx of mannitol and efflux of K^+ in addition to the influx of calcium.[114]

Pasteurella haemolytica leukotoxin

Pasteurella haemolytica is an important component of the bovine respiratory disease (BRD) complex and, as such, causes significant pneumonia and death in cattle.[115] P. haemolytica produces a toxin which is highly specific for ruminant leukocytes[116] and the detrimental effect of this leukotoxin on alveolar macrophages and PMNs is believed to contribute to the production of pneumonic lesions.[116,117] Slocombe et al.[118] demonstrated that the presence of neutrophils was required for the production of acute lung injury following the intratracheal inoculation of P. haemolytica. Calves which were neutrophil-depleted by the administration of hydroxyurea did not develop clinical signs or lung lesions after the adminstration of P. haemolytica. However, calves with normal levels of neutrophils developed acute clinical signs and severe lung lesions following inoculation of an identical suspension of P. haemolytica.[118] Proteolytic enzymes released from the granules of lysed phagocytes are believed to be responsible for the damage to pulmonary tissues and the extensive degenerative changes produced in the lung.[118,119,120] In more chronic cases, elimination of alveolar macrophages and PMNs may lead to decreased resistance to microbial infection and consequently more severe respiratory disease.[117,121]

The P. haemolytica leukotoxin damages bovine neutrophils,[115,116,119,121,122,123] lymphocytes,[123] blood mononuclear cells,[122,123] and alveolar macrophages.[117,122,123] It is also toxic

for PMNs from sheep, goats, and antelope.[116] No significant effect is produced on nonruminant leukocytes,[116,123] nor on erythrocytes from any of several ruminant and nonruminant species tested.[119,121,123] Optimal leukotoxin production occurs during the logarithmic phase of growth and decreases during the stationary phase.[115,117,119,121,122] The leukotoxin is heat-labile,[119,121] oxygen stable,[119,121] and released into broth culture supernatant.[116,117,119,123] The toxic effect is dose dependent[123] and leukotoxic activity is inactivated by trypsin,[119,121] chymotrypsin,[119] and protease.[119] As is the case with the E. coli alpha-hemolysin,[107,109,113] calcium is required for leukotoxin activity[124,125] and cannot be substituted for by magnesium,[124,125] manganese,[124] or zinc.[124] Extracellular leukotoxic activity was neutralized by convalescent serum from a natural case of P. haemolytica pneumonia[119] and post-infection sera from experimentally inoculated calves.[121] Neutralizing activity was also found in a serum pool from young cattle[119] and serum from normal adult cattle.[121]

Haemophilus pleuropneumoniae toxins

Haemophilus pleuropneumoniae is the causal agent of a severe and economically important respiratory disease in swine.[126,127,128,129] The rapid clinical course of the disease and the severe vascular changes which are produced by H. pleuropneumoniae suggest that a potent toxin(s) is produced early in the disease process.[128] Indeed, H. pleuropneumoniae is believed to elaborate at least two distinct toxins;

the heat-stable endotoxin and an extracellular hemolysin.[129] Both of these toxins are believed to contribute to the development of lung lesions.[129]

Investigations characterizing the H. pleuropneumoniae hemolysin have produced conflicting results. Nakai et al.[130] described a heat-stable extracellular hemolysin which was not inactivated by autoclaving at 121 C for two hours, nor by treatment with formalin, trypsin, or pronase. Results of partial purification by sucrose gradient ultracentrifugation indicate that the heat-stable toxin is a carbohydrate.[126] Dose and time dependent cytocidal and antiphagocytic effects were produced on porcine pulmonary macrophages by the heat-stable hemolysin.[126]

Other investigators have concluded that the extracellular hemolysin of H. pleuropneumoniae is RNA-dependent,[131] heat-labile,[127,131] and sensitive to degradation by pronase,[127,131] trypsin,[127,131] and chymotrypsin.[131] These characteristics suggest that the H. pleuropneumoniae hemolysin is a heat-labile protein.[127] Maximum hemolytic activity is produced by early to mid logarithmic phase H. pleuropneumoniae cultures.[127] Maudsley and Kadis[127] have concluded that H. pleuropneumoniae may produce two distinct hemolysins and different strains may be capable of producing either or both of these toxins.

Cell-free supernatants from H. pleuropneumoniae cultures, given intranasally to mice, produced severe pneumonia with extensive necrosis

and neutrophil infiltration.[129] The severe pulmonary lesions were attributed to a heat-labile component of the supernatant, perhaps the extracellular hemolysin, because heat-inactivated supernatant produced only mild lesions and minimal neutrophil infiltration. Viable H. pleuropneumoniae organisms have been demonstrated to be toxic for bovine neutrophils,[128] porcine neutrophils,[128] porcine pulmonary macrophages,[132] and porcine blood monocytes[132] in vitro. Although the relationship between the leukotoxic effect of H. pleuropneumoniae and the H. pleuropneumoniae hemolysin has not been determined, bacteria-free culture supernantants which were neutrophil-toxic were also toxic for porcine erythrocytes.[128] Both the neutrophil-toxic and hemolytic activity of cell-free supernatant are inhibited by oxygen and cholesterol and destroyed by trypsin and heat.[128] Toxicity of supernatant for leukocytes[128,132] and erythrocytes[128] was neutralized by specific antisera[128] and convalescent sera from naturally infected pigs.[128,132] A correlation was demonstrated between both cytotoxic activities and an increase in protein but not DNA or 2-keto-3-deoxyoctonate (KDO) in supernatants, indicating that the toxic product(s) is released from intact bacteria.[128] Although the leukotoxic and hemolytic activities of H. pleuropneumoniae share several characteristics in common, purification of the substance(s) is necessary before it can be determined whether one molecule is responsible for both of these activities.[128]

In vivo, a H. pleuropneumoniae toxin(s) which produces impaired

phagocytosis and cell death of pulmonary phagocytic cells would lead to decreased resistance to H. pleuropneumoniae infection[126] and enhanced survival of bacteria in the lung.[126,128] In addition, release of substances from dead and dying pulmonary macrophages[126] and neutrophils[129] could cause irritation,[126] fibrin deposition,[126] and contribute significantly to the disease process.[129]

Genetic relationships between toxins from gram negative bacteria

Extensive DNA homology has been demonstrated between the genetic determinants coding for hemolysin and/or leukotoxin production in several species of gram negative bacteria. Southern hybridization of DNA from hemolytic isolates of Proteus mirabilis,[133] Proteus vulgaris,[133,134] and Morganella morganii[133,134] to regions of the E. coli alpha-hemolysin determinant demonstrated clear but incomplete homology between genes coding for production of hemolysin in these four species of bacteria. Hemolytic and uropathogenic isolates of Proteus vulgaris and Morganella morganii express a polypeptide sequence similar in molecular size (110 kilodaltons) and antigenicity to the HlyA protein of E. coli alpha-hemolysin.[134] The 101.9-kilodalton P. haemolytica leukotoxin protein, LKTA, also shares extensive homology with the 109.8-kilodalton HlyA protein[135,136] and cross reacts with E. coli hemolysin antisera in Western blot analysis.[136] In addition, the 19.8-kilodalton P. haemolytica leukotoxin protein, LKTC, shares extensive homology with the 19.7-kilodalton HlyC alpha-hemolysin

protein.[135,136] These significant homologies suggest a common origin for the genes involved in production of these toxins.[136]

The *E. coli* alpha-hemolysin determinant is believed to be of non-*E. coli* origin.[133,135] The mobile nature of the hemolysin, which is present in chromosomal[137,138] as well as extrachromosomal locations,[138,139,140,141] would have allowed aquisition of the hemolysin from another species of bacteria. Origin of alpha-hemolysin in an organism not closely related to *E. coli* is indicated by ultilization of rare *E. coli* codons by alpha-hemolysin in a pattern unlike that of other *E. coli* genes[135] and a much lower guanidine plus cytosine content than genomic *E. coli* DNA.[133,135] A common ancestry with the hemolysin determinant of *Proteus* is suggested by the extensive DNA homology shared by these two toxins[133,134] and the low guanidine plus cytosine content of alpha-hemolysin,[133,135] which is very close to that of *Proteus mirabilis* and *Proteus vulgaris* genomic DNA.[133]

COMPARATIVE CHARACTERIZATION OF THE LEUKOCIDIC AND HEMOLYTIC ACTIVITY OF *MORAXELLA BOVIS*

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SUMMARY

The cytotoxic effect of *Moraxella bovis* 118F on bovine polymorphonuclear leukocytes (PMNs) was evaluated and characterized using a ^{51}Chromium release assay. PMNs harvested from normal adult cattle were labeled with ^{51}Cr. The leukocidic activity produced by *M. bovis* 118F, a hemolytic strain of *M. bovis*, was heat-labile. A live culture of this strain, at a ratio of 100 bacteria/PMN, released 97.7% of the ^{51}Cr from labeled PMNs. Neither a heat-killed preparation of *M. bovis* 118F nor a live or heat-killed preparation of *M. bovis* IBH63 (a nonhemolytic and nonpathogenic strain) produced significant release of ^{51}Cr.

M. bovis 118F broth culture filtrates prepared for evaluation of leukocidic activity were also evaluated for hemolytic activity. These two toxic activities were demonstrated to have several characteristics in common. Both are filterable, heat-labile, produced by a hemolytic strain, and are released during early logarithmic phase growth from broth cultures. Both toxic activities are protected from degradation by phenylmethyl-sulfonyl fluoride, a serine protease inhibitor. Leukocidic and hemolytic activity are both dependent on the presence of calcium ions; a dependence for which magnesium ions cannot substitute. Filtrate produced 54.1% ^{51}Cr release from labeled PMNs and contained 646.7 hemolytic units/ml respectively when saline + 10 mM $CaCl_2$ was used as diluent. Neither saline nor saline + 10 mM $MgCl_2$ supported

leukocidic or hemolytic activity. Convalescent sera, collected from several calves 10 to 38 days after M. bovis infection, were shown to significantly neutralize leukocidic and hemolytic activity when compared to pre-infection serum samples. In addition, there was no significant difference between the extent neutralization of leukocidic and hemolytic activity produced by specific immune sera. These common characteristics indicate that the putative leukocidin of M. bovis may be the previously described hemolysin.

INTRODUCTION

Moraxella bovis is the etiologic agent of infectious bovine keratoconjunctivitis (IBK).[1,2,3] Even though IBK was estimated in 1984 to cause losses greater than 200 million dollars anually to cattle producers in the United States,[4] the pathogenesis of this economically important disease is not well understood. Numerous virulence factors have been implicated in the pathogenesis of IBK. The hemolysin of M. bovis has been characterized[5,6,7] and is considered to be a virulence factor.[8,9,10] The production of cytotoxic substances[11,12] by M. bovis has been described. The relationship between the hemolysin and cytotoxic substance(s) produced by M. bovis has not been established.

Collagenase has been purified from human polymorphonuclear leukocytes.[13,14,15] This enzyme has been found to be localized in the specific granules of neutrophils and would be released during phagocytosis or when the neutrophils are subjected to other stimuli.[15] Activated collagenases, released into an area of inflammation, could lead to further localized tissue damage.[14] Polymorphonuclear leukocytes, in fact, have been demonstrated to be directly involved in the development of corneal lesions. PMNs have been shown to be responsible for the development of corneal ulcers,[16,17] delayed corneal wound healing,[18] and corneal edema and opacification.[19,20] Lysosomes harvested from PMNs, containing collagenases and other proteolytic enzymes, have been shown to be the component of the cell responsible

for these corneal injuries.[18,21]

Large numbers of neutrophils infiltrate the corneas of cattle with natural or experimental IBK infection.[22,23,24] These neutrophils are closely associated with M. bovis in the corneal lesions and have been described to be in various stages of degeneration and degranulation.[22,23,24] Since M. bovis does not produce a collagenase,[25] the abundant neutrophils present would be a likely source for this important enzyme and could contribute significantly to lesion development. Observations by several investigators support this hypothesis. Severe corneal lesions have been reported to occur in areas where few, if any, Moraxella bovis organisms but abundant inflammatory cells have been observed.[22,23,24,26]

A leukocidin produced by M. bovis, causing direct damage to neutrophils, would result in extensive release of neutrophil enzymes which would be expected to contribute significantly to the corneal damage produced during IBK. For this reason, the presence of a M. bovis leukocidin was investigated. Because only hemolytic strains of M. bovis are capable of producing IBK[10,24] and its associated severe corneal lesions, the relationship between the leukocidic and hemolytic activity of M. bovis was also investigated.

MATERIALS AND METHODS

Bacterial Cultures

M. bovis 118F was isolated from a naturally occurring case of infectious bovine keratoconjunctivitis (IBK) and has been shown to be pathogenic for cattle.[27] M. bovis IBH63 was obtained from G. W. Pugh (National Animal Disease Center, Ames, IA). This organism is a nonhemolytic and nonpathogenic variant of a hemolytic strain.[10] A suspension of M. bovis 118F or M. bovis IBH63 was prepared in 0.85% saline + 10 mM $MgCl_2$ from a culture grown on 5% bovine blood agar (5% defibrinated bovine blood; tryptose blood agar base, Difco Laboratories, Detroit, MI) for 13 hours and standardized to an optical density of 0.400 at 600 nm. A 1% inoculum was added to 200 ml of tryptose-bovine serum albumin (BSA) broth (1% tryptose, Difco Laboratories, Detroit, MI; 0.5% NaCl, Fischer Scientific, Pittsburg, PA; 0.5% bovine serum albumin, Sigma Chemical Corp., St. Louis, MO) and the broth culture was incubated for 4.5 hours at 37 C with shaking (100 rpm with 500 ml Erlenmeyer flasks, Aquatherm waterbath shaker, New Brunswick Scientific Co. Inc., Edison, NJ). The bacteria were harvested by centrifugation at 2000 x g for 20 minutes, washed three times in saline + 10 mM $MgCl_2$, and resuspended in saline + 10 mM $MgCl_2$ so that a 1:10 dilution had an optical density of 0.200 at 600 nm (approximately 5×10^9 bacteria/ml). This concentration of bacteria

was used in all assays unless otherwise noted. As required, bacterial suspensions prepared as described were killed by heating at 56 C for one hour. Purity of the live M. bovis cultures and sterility of the heat-killed preparations were ascertained by culture.

Filtrate Preparation

A suspension of M. bovis 118F was prepared in 0.85% saline + 10 mM $MgCl_2$ from a 13 hour bovine blood agar plate culture and standardized to an optical density of 0.800 at 600 nm. A 1% inoculum of a 1:10 dilution was added to 200 ml tryptose-BSA broth. The broth culture was incubated at 37 C with shaking for 4 to 5 hours until a final optical density of 0.030 at 600 nm was reached. The culture was then filtered through a 400 nm polycarbonate filter (Nagle Co., Rochester, NY). Forty microliters of a phenylmethyl-sulfonyl fluoride (PMSF) solution (100 mM in methanol, stored at -20 C, Sigma Chemical Corp., St. Louis, MO) were added per 10 ml of filtrate unless otherwise noted. The filtrate was held at 4 C or heated at 56 C for one hour before evaluation for leukocidic or hemolytic activity in the appropriate assay. The filtrate was prepared fresh for each use and cultured to ascertain sterility.

Polymorphonuclear Leukocyte (PMN) and Red Blood Cell (RBC) Preparation

Venous blood was collected from normal adult Holstein steers into acid citrate dextrose solution or Alsever's solution for PMN or RBC preparation respectively. PMNs were isolated as described.[28] Briefly, after centrifugation the plasma, buffy coat layer, and upper portion of the RBC layer were removed by aspiration. The remaining RBCs were lysed twice using phosphate buffered deionized water and isotonicity was restored using phosphate buffered 2.7% NaCl. The PMNs were pelleted by centrifugation and standardized to a concentration of 2.5 x 10^7 cells/ml in Medium 199 (M199, Gibco Laboratories, Grand Island, NY) + 5% fetal bovine serum (FBS, J. R. Scientific, Woodland, CA). RBCs in Alsever's solution were stored for no longer than 10 days at 4 C. Cells were washed three times in saline and resuspended in saline + 10 mM $CaCl_2$ to a 1% solution on the day of use.

Labeling of PMNs

PMNs were labeled with ^{51}Chromium (Amersham Corp., Arlington Heights, IL) using a modification of a previously described method.[29] ^{51}Chromium (2 uCi/10^6 PMNs) was added to PMNs and the mixture incubated at 37 C with 5% CO_2 for 30 minutes. The PMNs were sedimented at 200 x g for 10 minutes and washed three times in phosphate-buffered saline solution (PBSS; pH = 7.4). The PMNs were resuspended in M199 + 5% FBS

to a concentration of 5.0×10^7 cells/ml unless otherwise noted.

Neutrophil Toxicity Assay

Test wells were prepared in triplicate for each animal's PMNs. Fifty microliters *M. bovis* (2.5×10^8 organisms) or 100 ul *M. bovis* broth culture filtrate were added to 100 ul M199 + 5% FBS in 96 well U-bottom micro plates (Costar, Cambridge, MA). The reaction was started by the addition of 50 ul ^{51}Cr labeled PMNs (2.5×10^6 cells). Standards, representing maximal release of ^{51}Cr from PMNs, were prepared by adding 150 ul of a 1% Triton-X 100 (Fischer Scientific, Fair Lawn, NJ) solution to 50 ul PMNs. Backgrounds, representing spontaneous release of ^{51}Cr from PMNs, were prepared by substituting 50 ul saline + 10mM $MgCl_2$ or 100 ul tryptose-BSA broth + PMSF for bacteria and filtrate respectively. Standards and backgrounds were prepared in triplicate for each animal's PMNs. The micro plates were incubated at 37 C with 5% CO_2 for 60 minutes (bacteria) or 180 minutes (filtrate). ^{51}Cr released into the supernatant was harvested using a SCS harvesting press and frames (Skatron Inc., Sterling, VA). The collected supernatant was counted for two minutes in a gamma counter (Biogamma II, Beckman Instruments Inc., Palo Alto, CA), the mean determined for each triplicate sample, and the percent ^{51}Cr release determined using

the following formula;

$$\% \text{ release} = \frac{\text{mean count test} - \text{mean count background}}{\text{mean count standard} - \text{mean count background}} \times 100$$

The neutrophil toxicity assay was modified to evaluate the effects of concentration and time on ^{51}Cr release. Serial two fold dilutions of a live M. bovis 118F suspension, which originally contained 5×10^9 bacteria/ml, were made in saline + 10 mM $MgCl_2$. Dilutions of this suspension through 1:16 were added to a constant concentration of PMNs (5×10^7/ml) and evaluated in the standard assay. M. bovis 118F suspensions containing 100 bacteria/PMN and 10 bacteria/PMN were used in the standard assay and the released ^{51}Cr was harvested at 5, 15, 30, 45, 60, and 120 minutes. Saline, saline + 10 mM $CaCl_2$, and saline + 10 mM $MgCl_2$ were substituted for M199 + 5% FBS as diluent and for resuspending ^{51}Cr labeled PMNs in some experiments.

Hemolysin Assay

The hemolytic activity of M. bovis 118F broth culture filtrate was evaluated by modification of previously described methods.[6,7] Briefly, serial two-fold dilutions of 1.5 ml of broth culture filtrate were made to 1:1024 in saline + 10 mM $CaCl_2$ in 12-well plastic test plates (Costar, Cambridge, MA). An equal volume of a 1% bovine RBC suspension in saline + 10 mM $CaCl_2$ was added to each well.[6] A control well

contained 1.5 ml Tryptose-BSA broth, 3 ul PMSF, and 1.5 ml of the 1% bovine RBC suspension. Plates were incubated at 37 C with 5% CO_2 for three hours and the contents of each well were centrifuged at 1500 x g to pellet any remaining RBCs.[6] The optical density of the supernatant containing released hemoglobin was determined at 540 nm. The number of hemolytic units per ml was determined from a standard curve[7] prepared in duplicate at each assay time. One hemolytic unit is that quantity of hemolysin which will produce 1% lysis of the standard RBC suspension.

Neutralization of Leukocidic and Hemolytic Activity

Paired pre and post-*M. bovis* 118F infection sera from three calves were evaluated for their ability to neutralize the leukocidic and hemolytic activity of *M. bovis*. All calves used in this study were colostrum deprived and isolation reared, and were infected with *M. bovis* as described.[27] Sera from calves 82, 87, and 97 were collected before infection and 38, 28, and 10 days post-infection respectively. All sera were heat-inactivated at 56 C for 30 minutes and diluted 1:40 in 0.85% saline + 10 mM $CaCl_2$. Two parts *M. bovis* broth culture filtrate were combined with one part 1:40 serum and incubated in a 37 C waterbath for 30 minutes. The filtrate-serum suspensions were evaluated in the neutrophil toxicity and hemolysin assays. Saline + 10 mM $CaCl_2$ was substituted for M199 + 5% FBS as diluent and for

resuspending labeled PMNs in the neutrophil toxicity assay. The hemolysin assay was performed using 1.5 ml of the filtrate-serum suspension. Control wells contained one part 1:40 serum, two parts Tryptose-BSA broth, and an equal volume of the 1% bovine RBC suspension.

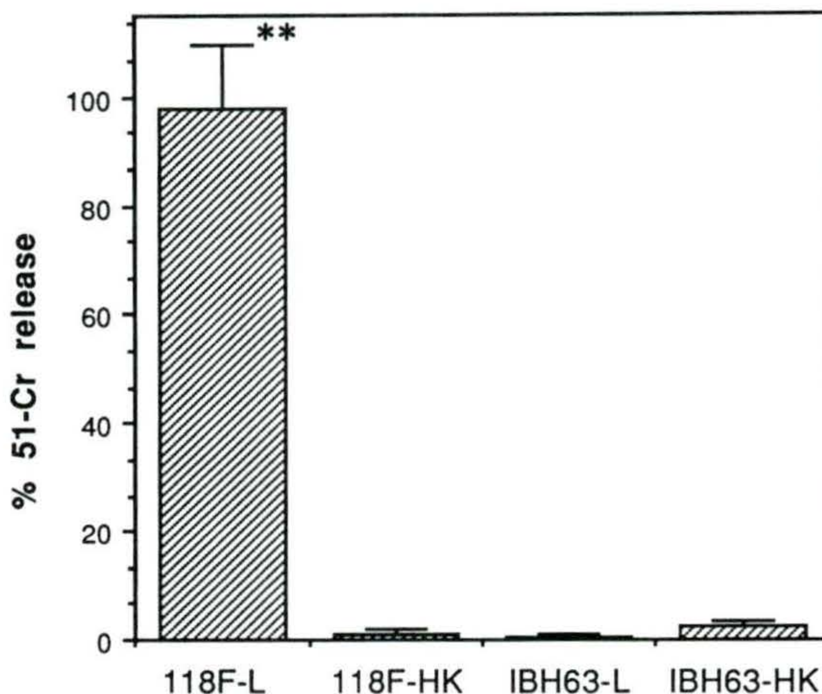
Statistical Analysis

Numerical data were subjected to analysis of variance procedures and neutrophil toxicity data were blocked for animal differences. The significance of differences between treatment means was determined in all cases by least significant difference t tests ($P \leq .01$).

RESULTS

A heat-labile leukocidic activity was produced by a hemolytic strain of Moraxella bovis. This was demonstrated by release of 97.7% (± SEM 11.8) of ^{51}Cr from labeled PMNs incubated with live M. bovis 118F organisms (Figure 1). The leukocidic activity was completely destroyed by heating the bacterial suspension at 56 C for 1 hour. Neither live nor heat-killed suspensions of M. bovis IBH63 produced significant release of ^{51}Cr from PMNs. Because bacterial suspensions other than live M. bovis 118F did not produce significant release of ^{51}Cr, all further characterization of the leukocidic activity involved the use of live M. bovis 118F organisms or filtrate prepared from broth cultures of this strain.

The effects of concentration and time on leukocidic activity were examined. A live M. bovis suspension which contained 100 bacteria/PMN released 80.7% (± SEM 2.9) of the ^{51}Cr from labeled PMNs. A value of 40.5% (± SEM 2.5) was obtained when there were approximately 6.25 bacteria/PMN. As the ratio of bacteria to PMN decreased by dilution of the bacterial suspension, the percent release of ^{51}Cr decreased porportionally (data not shown). The percent release of ^{51}Cr was determined after live M. bovis organisms were incubated with PMNs for various periods of time. The bacteria released 34.0% (± SEM 3.7) and 18.4% (± SEM 4.3) of the ^{51}Cr after 5 minutes incubation with 100:1 and 10:1 bacteria/PMN respectively. The percent ^{51}Cr release increased



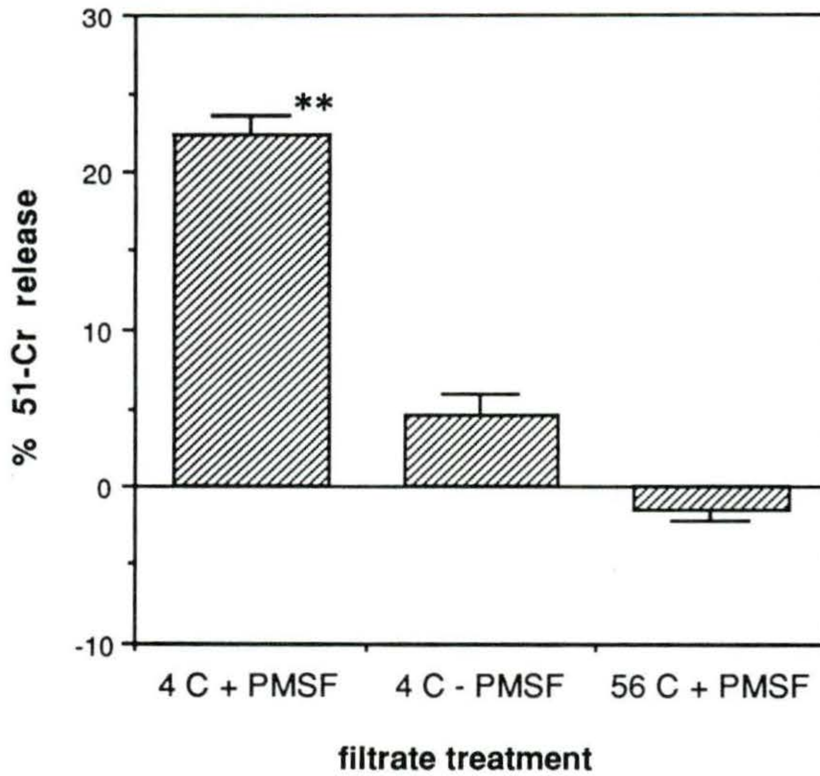
**p <.01.

Figure 1. Leukocidic activity of live and heat-killed (56 C, 1 hour) Moraxella bovis suspensions. 118F-L - live M. bovis 118F; 118F-HK - heat-killed M. bovis 118F; IBH63-L - live M. bovis IBH63; IBH63-HK - heat-killed M. bovis IBH63. Leukocidic activity is expressed as mean percent (+ SEM) ^{51}Chromium release from labeled bovine neutrophils collected from four donors

with the length of incubation (data not shown) and values after 120 minutes incubation were 96.5% ($\pm$ SEM 10.0) and 63.9% ($\pm$ SEM 10.7) for 100:1 and 10:1 bacteria/PMN respectively.

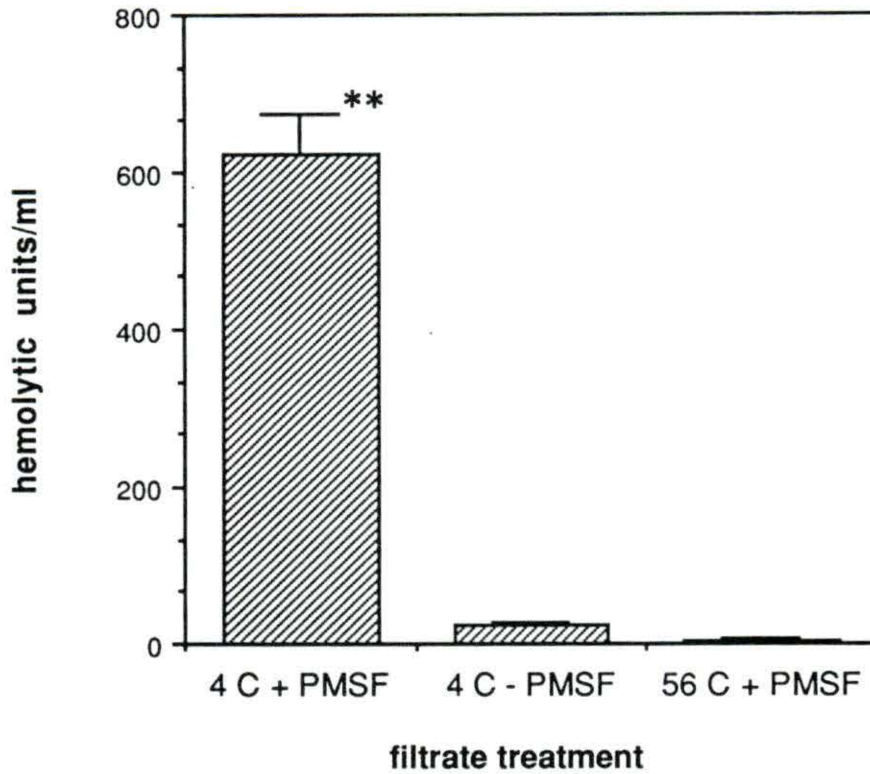
The presence of leukocidic and hemolytic activity in filtrate prepared from broth cultures was evaluated. Both leukocidic and hemolytic activity were present in filtrate from log phase broth cultures and were lost in stationary phase cultures (data not shown). A significant amount of leukocidic and hemolytic activity was lost when filtrates were prepared using any type of membrane other than one made of polycarbonate (data not shown).

The leukocidic and hemolytic activity of filtrates with and without PMSF were compared. When PMSF was added and the filtrate was held at 4 C for 60 minutes, 22.3% ($\pm$ SEM 1.3) of the ^{51}Cr was released (Figure 2a). Significantly less ^{51}Cr was released from labeled PMNs when a portion of the same filtrate was held at 4 C for 60 minutes without added PMSF (4.5% $\pm$ SEM 1.5) and when filtrate with PMSF was heated at 56 C for 60 minutes (-1.5% $\pm$ SEM 0.72). Filtrate held at 4 C for 60 minutes to which PMSF had been added contained 624.0 ($\pm$ SEM 51.2) hemolytic units/ml (Figure 2b). Filtrate held at 4 C for 60 minutes without PMSF contained only 23.8 ($\pm$ SEM 3.8) hemolytic units/ml and filtrate + PMSF held at 56 C for 60 minutes contained 0.0 hemolytic units/ml. Leukocidic and hemolytic activity were preserved in filtrate preparations by the addition of PMSF and holding the filtrate at 4 C before use. Both activities were completely destroyed by heating at



**p <.01.

Figure 2a. Effects of PMSF and temperature on leukocidic activity of M. bovis 118F broth culture filtrate. 4 C + PMSF - filtrate held at 4 C for one hour before use with PMSF; 4 C - PMSF - filtrate held at 4 C for one hour before use without PMSF; 56 C + PMSF - filtrate held at 56 C for one hour before use with PMSF. Leukocidic activity is expressed as mean percent (± SEM) [51]Chromium release from labeled bovine neutrophils collected from four donors



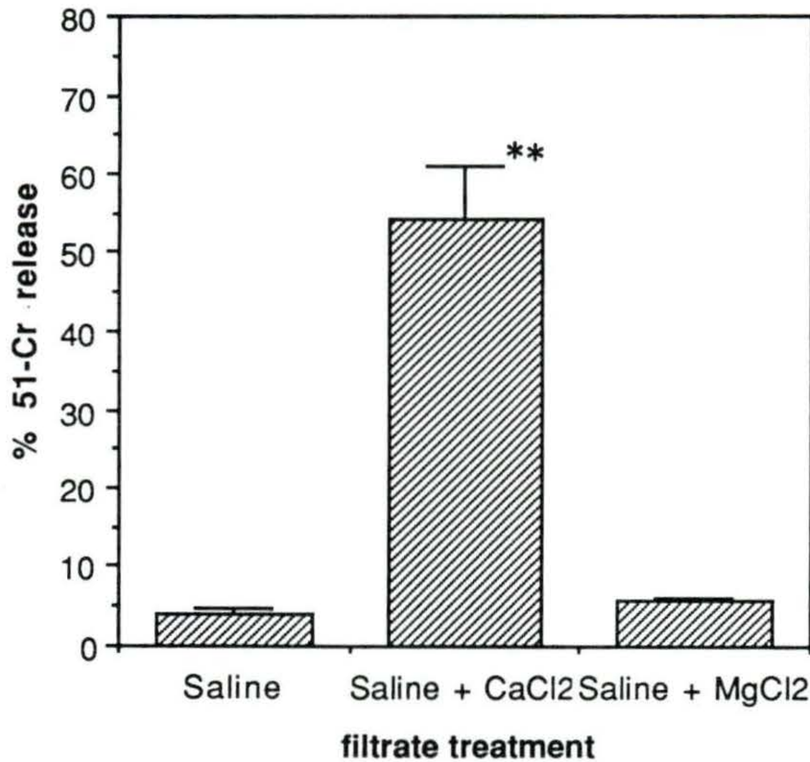
**p <.01.

Figure 2b. Effects of PMSF and temperature on hemolytic activity of M. bovis 118F broth culture filtrate. 4 C + PMSF - filtrate held at 4 C for one hour before use with PMSF; 4 C - PMSF - filtrate held at 4 C for one hour before use without PMSF; 56 C + PMSF - filtrate held at 56 C for one hour before use with PMSF. Hemolytic activity is expressed as the mean (± SEM) hemolytic units/ml filtrate of four hemolysin assays.

56 C for 1 hour. Sensitivity to heat inactivation (at 56 C) of both toxic activities was maintained in the presence of PMSF.

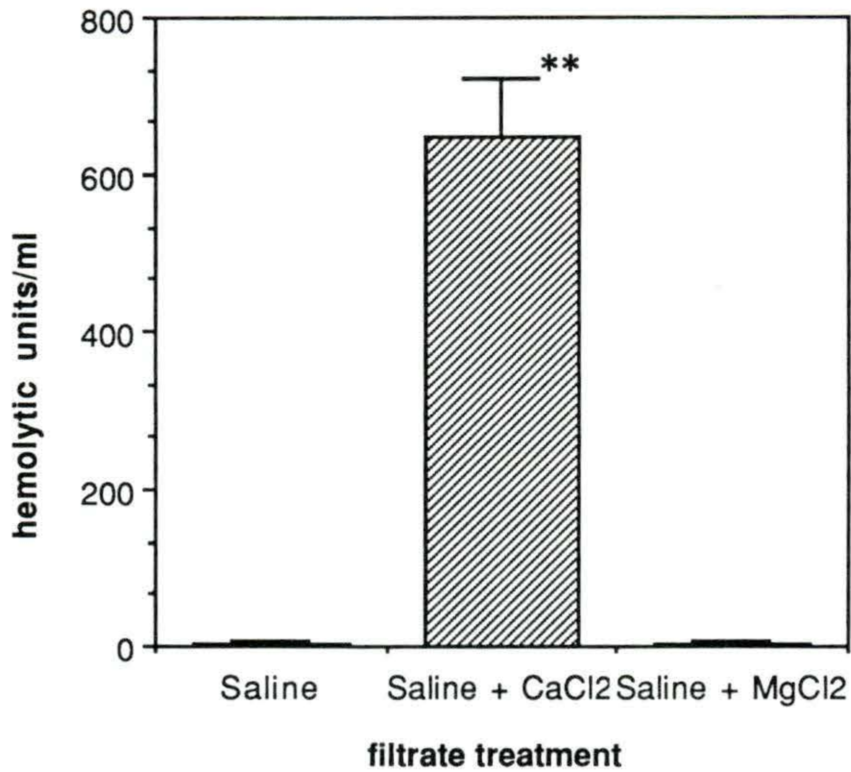
The dependence of the leukocidic activity of whole M. bovis 118F organisms and filtrate on the presence of calcium and/or magnesium was determined. Saline + 10 mM $CaCl_2$ supported the leukocidic activity of whole organisms as demonstrated by a ^{51}Cr release value of 38.8% (± SEM 4.8). Saline + 10 mM $MgCl_2$ and saline alone did not support this activity with values of -0.88% (± SEM 0.42) and -0.45% (± SEM 0.53) respectively. The leukocidic activity of filtrate was shown to be dependent on the presence of $CaCl_2$ with 54.1% (± SEM 7.0) ^{51}Cr release obtained with 10 mM $CaCl_2$ in the diluent (Figure 3a). Values obtained when saline or saline + 10 mM $MgCl_2$ were used as diluent were 4.2% (± SEM 0.7) and 5.6% (+ SEM 0.5) ^{51}Cr release respectively. These values are not significantly different from each other, indicating that saline + $MgCl_2$ is not capable of supporting leukocidic activity. Filtrate contained 646.7 (± SEM 75.3) hemolytic units/ml when saline + 10 mM $CaCl_2$ was used as diluent (Figure 3b). Saline alone and saline + 10 mM $MgCl_2$ did not support hemolytic activity. These results demonstrate that leukocidic and hemolytic activity are dependent on the presence of calcium ions; a dependence for which magnesium ions cannot substitute.

The ability of various sera to neutralize the leukocidic and hemolytic activity of M. bovis 118F filtrate was evaluated. All serum samples were diluted 1:40 to eliminate nonspecific inactivation of toxic activity produced by undiluted serum. Neutralization was



**p <.01.

Figure 3a. Effects of calcium and magnesium in assay diluent on leukocidic activity of M. bovis 118F broth culture filtrate. Saline - 0.85% NaCl solution; Saline + $CaCl_2$ - 0.85% NaCl solution with 10 mM $CaCl_2$; Saline + $MgCl_2$ - 0.85% NaCl solution with 10 mM $MgCl_2$. Leukocidic activity is expressed as mean percent (± SEM) ^{51}Chromium release from labeled bovine neutrophils collected from eight donors



**p <.01.

Figure 3b. Effects of calcium and magnesium in assay diluent on hemolytic activity of M. bovis 118F broth culture filtrate. Saline - 0.85% NaCl solution; Saline + $CaCl_2$ - 0.85% NaCl solution with 10 mM $CaCl_2$; Saline + $MgCl_2$ - 0.85% saline + 10 mM $MgCl_2$. Hemolytic activity is expressed as the mean (± SEM) hemolytic units/ml filtrate of four hemolysin assays

expressed as the percent reduction in leukocidic or hemolytic activity produced by post-*M. bovis* infection sera using the paired pre-infection serum as a reference. Post-*M. bovis* infection sera from calves 82, 87, and 97 neutralized the leukocidic and hemolytic activity of filtrate (Figure 4). This was demonstrated by a significant reduction in ^{51}Cr release from labeled PMNs and hemolytic units/ml respectively, when filtrate was pre-incubated with convalescent sera. There was no significant difference ($p > .05$) between the percent neutralization of leukocidic and hemolytic activity produced by the convalescent serum of each calf.

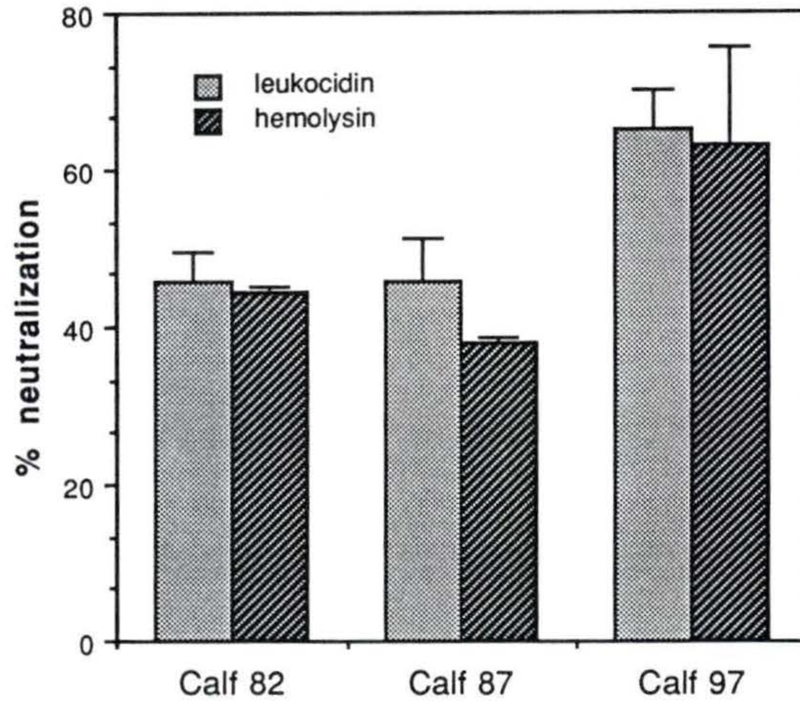


Figure 4. Neutralization of leukocidic and hemolytic activity of M. bovis 118F broth culture filtrate by post-M. bovis infection sera from three calves. Percent neutralization is expressed as the percent reduction (± SEM) in leukocidic or hemolytic activity using the paired pre-infection serum as a reference. For determination of leukocidic and hemolytic activity, eight neutrophil donors and four hemolysin assays were used respectively

DISCUSSION

A putative leukocidin produced and secreted by M. bovis has been described. The identification of this leukocidin, which causes significant damage to neutrophils and would be expected to increase the release of lysosomal enzymes, should contribute greatly to our understanding of the pathogenesis of IBK. This leukocidin could not only contribute indirectly to corneal lesions by its damaging effect on neutrophils; but it could also directly enhance the colonization of M. bovis in the eye and subsequent length of disease. Damaged neutrophils would be ineffective in clearing M. bovis organisms, leading to increased numbers of viable bacteria in the eye.

Previous reports have briefly described the cytotoxic effect of M. bovis on various host cells.[11,12] ^{51}Chromium release values from labeled neutrophils incubated with live 24 hour culture suspensions of M. bovis[12] were comparable with those obtained with live whole cell suspensions of M. bovis 118F in the present study. However, Kagonyera et al.[12] did not demonstrate cytotoxic effects with filtrates of sonicated 24 hour cultures. This is not surprising, as little cell-free leukocidic activity would be expected to be present in cultures at this late stage of growth. A cytotoxin which is able to kill large numbers of bovine monocytes and goat macrophages has been described to be produced by virulent M. bovis isolates.[11] Viability of the bacteria was necessary to cause a maximal cytotoxic effect and heat-killed

organisms were essentially devoid of cytotoxic activity.[11,12] The relationship between the leukocidic activity of *M. bovis* described in our study and this cytotoxin(s) must be further defined.

Hemolysin production is considered to be a major virulence factor of *M. bovis*[8,9,10] and only hemolytic strains of the organism are capable of producing IBK.[10,24] The hemolysin of *M. bovis* has been characterized by several investigators.[5,6,7] The exact relationship between the hemolysin and the leukocidic activity of *M. bovis* described in our study is not known. However, these two toxic activities share several characteristics in common, which indicates that both activities may be the result of the action of one toxin. Conclusive proof awaits either the isolation of the substance(s) or comparison of the cloned gene(s) involved in production of the toxin(s).

Characteristics of the *M. bovis* leukocidic activity demonstrated in our study include the ability to react quickly with bovine neutrophils and to produce a dose dependent cytotoxic effect. Characteristics shared with the *M. bovis* hemolysin include; production by a hemolytic strain, destruction of activity by heating at 56 C for 1 hour,[5,6,7] and separation of activity from whole cells by filtration.[6,7] Several reports describe production of the hemolysin only during logarithmic phase growth.[6,7] Similarly, leukocidic activity is present in log phase but absent in stationary phase cultures. Proteases produced by *M. bovis* and secreted into the broth culture medium are perhaps at least partially responsible for the

decrease in both activities observed in later stage cultures. Previously, the hemolysin was found to be very labile to routine procedures of concentration and purification[7] and it was concluded that this instability may be due to production of extracellular proteolytic enzymes.[30] Sufficient proteases may not have been produced and secreted by M. bovis in log phase cultures to totally destroy extracellular leukocidic and hemolytic activity, but levels of these enzymes would be expected to increase with the length of incubation. As is believed to be the case with the E. coli alpha-hemolysin, perhaps there is an inhibitory mechanism which decreases synthesis in later stages, and the toxin(s) already formed is destroyed by proteases.[31]

Hemolytic and leukocidic activity of filtrate is protected by the serine protease inhibitor PMSF. The addition of PMSF to early log phase culture filtrates is believed to preserve both of these activities by helping to inhibit M. bovis serine proteases. Previous attempts to stabilize the hemolysin in filtrates by the addition of various protease inhibitors have been unsuccessful.[6] The successful use of PMSF in the current study demonstrated that use of a specific protease inhibitor was necessary to protect hemolytic and leukocidic activity.

Our study found that leukocidic and hemolytic activity are both dependent on the presence of calcium. This dependence was demonstrated to be specific for calcium and another divalent cation, magnesium, could not be substituted. All previous reports do not agree that

hemolysin activity is calcium dependent. They do agree, however, that the activity of the hemolysin is enhanced by the presence of divalent cations.[5,6,7] The reason for the discrepancy between various reports concerning calcium dependence is not clear. In one report,[7] the values for hemolytic activity were very low in all groups; allowing little room for comparison between treatments. The media used for growth of *M. bovis* in those studies which did not show a dependence on calcium may have contained a small amount of calcium; sufficient for hemolysin activation without additional calcium being added to the assay. This was found to be the case when the dependence of the *E. coli* hemolysin on calcium was in question.[32]

Both hemolytic and leukocidic activity were neutralized to the same extent by post-*M. bovis* infection sera from three different animals. This indicates that all animals responded to the same extent to these toxins and perhaps were, in fact, responding to the same antigen. The fact that the hemolytic and leukocidic activity of filtrate can be significantly neutralized by specific immune sera indicates that the toxin(s) is either excreted or is located on the outer membrane and is readily accessible for processing by the immune system. Vaccination with a preparation capable of stimulating the production of specific neutralizing antibody presents exciting possibilities for help in controlling this economically important disease. Antibody capable of neutralizing the leukocidic acitivity of *M. bovis* would protect neutrophils in the corneal stroma and prevent

their destruction and subsequent release of proteolytic enzymes. Less corneal edema, opacity, stromal liquefaction, and less inhibition of corneal wound healing would be produced due to the decrease in enzyme release. Antibody directed specifically against the leukocidic activity of *M. bovis* would also enhance bacterial phagocytosis and killing. This would decrease the colonization of the eye and the duration of the disease process.

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GENERAL DISCUSSION

Moraxella bovis 118F produces a rapid and dose dependent leukocidic effect on bovine neutrophils. Comparative characterization of the *M. bovis* hemolysin and this leukocidic activity revealed many shared properties. In addition, treatments which affected leukocidic activity were demonstrated to produce parallel effects on hemolytic activity. The results of the current study agree with previous studies characterizing the *M. bovis* hemolysin[53,54,55] and leukocidic activity.[69] Both toxic activities are present in early logarithmic phase broth culture filtrates[53,54,55,69] and are dependent on the presence of calcium ions.[53,69] Leukocidic and hemolytic activity are both heat-labile[53,54,55,69] and protected from degradation by the addition of phenylmethyl-sulfonyl fluoride (PMSF); properties which indicate that the toxin(s) is protein in nature. The results of Kagonyera et al.[69] and the current study demonstrate that leukocidic activity is associated with hemolytic strains, while nonhemolytic strains produce neither activity. Post-*M. bovis* infection sera neutralized extracellular leukocidic and hemolytic activity to the same extent, indicating that identical responses were produced to these antigens, or more significantly, perhaps the response was to a single antigen.

Disruption of the hemolysin gene by transposon mutagenesis could provide evidence that the hemolytic and leukocidic activities of *M.*

bovis are due to the actions of one toxin. Mutants with a single copy of the transposon in their genome and a demonstrated loss of hemolysis on blood agar would be evaluated for leukocidic and hemolytic activity. It could be tentatively concluded that both activities are due to the action of one gene product if the selected mutants were demonstrated to have lost both hemolytic and leukocidic activity. Final proof would be provided by isolation of the substance(s) or cloning of the specific genes involved in their production.

Comparison of the toxic activity of M. bovis filtrates in our study to E. coli alpha-hemolysin, P. haemolytica leukotoxin and H. pleuropneumoniae extracellular toxin reveals several characteristics shared by these gram negative bacterial toxins. All four toxins are produced during log phase growth,[95,105,108,117,121,127] are heat-labile,[93,121,127,128,131] excreted,[94,105,108,117,123,127,128] neutralized by specific immune sera,[93,119,121,128] and inactivated by proteases.[93,119,127,131] In addition, the M. bovis, E. coli,[107,109,113] and P. haemolytica[124,125] toxins are calcium dependent. Although not performed in the current study, the destruction of the M. bovis hemolysin,[53,54,55] E. coli alpha-hemolysin,[93,107,109] P. haemolytica leukotoxin,[119,121] and H. pleuropneumoniae extracellular toxin[127,128,131] by trypsin is well documented. The proteinacious nature of these toxins is indicated by their heat-lability and their susceptibility to inactivation by trypsin and proteases. Leukocytes and erythrocytes are damaged by M. bovis

culture filtrates, E. coli alpha-hemolysin[93,94,95,96] and H. pleuropneumoniae culture supernatants.[128]

Several gram negative bacterial toxins share extensive DNA homology in addition to physical characteristics. The significant homologies which exist between the DNA of the E. coli, Proteus mirabilis, Proteus vulgaris, Morganella morganii, and Pastuerella haemolytica toxins[133,134,135,136] indicates these toxin genes may share a common ancestry.[136] Investigation of the extent of DNA homology which exists between the M. bovis toxin(s) and representative gram negative toxins should be undertaken in light of the numerous properties shared by these toxins. Isolation of the M. bovis toxin(s) must first be accomplished before such a comparison can be made.

The leukocidic activity of M. bovis may contribute to the production of clinical IBK by two mechanisms. This toxin may enable M. bovis to resist attack and subsequent destruction by phagocytes. At sublethal concentrations of toxin, premature activation of neutrophils may result in release of granular enzymes and reduced bacteriocidal capacity. Neutrophils, severely damaged by lethal concentrations of toxin, would be ineffective in phagocytizing and killing M. bovis organisms, leading to increased proliferation and colonization. At both sublethal and lethal concentrations of toxin, the ability of M. bovis to establish and maintain an infection would be enhanced; leading to a prolonged and more severe disease process.

Neutrophils, prematurely activated or damaged by M. bovis, would

release proteolytic enzymes which may contribute to the development of IBK lesions. The release of enzymes, especially collagenases, would result in destruction and disruption of collagen fibrils[90,91] and would contribute significantly to corneal opacity[71] and ulceration.[87,88,89] The presence of degranulating neutrophils and the absence of significant numbers of _M. bovis_ organisms in severe lesions[27,30,31,32] provides strong evidence for the contributory role of lysosomal enzymes in IBK lesion production.

In the current study, convalescent sera from three experimentally infected calves neutralized both hemolytic and leukocidic activity of _M. bovis_ filtrate. These results indicate that the toxin(s) are readily accessible to the immune system and, at least in theory, an effective vaccine could be produced. Vaccination with a preparation which stimulates the production of high levels of leukocidin-neutralizing and opsonizing antibodies would protect neutrophils in the corneal stroma from damage and would facilitate uptake and intracellular killing of _M. bovis_ by phagocytic cells. Reduced numbers of bacteria and less damage to neutrophils would likely decrease the duration of ocular disease by allowing more rapid repair of less severely damaged tissues. The severe economic losses _M. bovis_ infection currently causes, due primarily to decreased feed efficiency and milk production,[1,2] would be significantly decreased by the development of an effective vaccine.

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