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# Complexities of the pathogenesis of *Mannheimia haemolytica* and *Haemophilus somnus* infections: challenges and potential opportunities for prevention?

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## Abstract

Progress in producing improved vaccines against bacterial diseases of cattle is limited by an incomplete understanding of the pathogenesis of these agents. Our group has been involved in investigations of two members of the family Pasteurellaceae, *Mannheimia haemolytica* and *Haemophilus somnus*, which illustrate some of the complexities that must be confronted. Susceptibility to *M. haemolytica* is greatly increased during active viral respiratory infection, resulting in rapid onset of a severe and even lethal pleuropneumonia. Despite years of investigation, understanding of the mechanisms underlying this viral–bacterial synergism is incomplete. We have investigated the hypothesis that active viral infection increases the susceptibility of bovine leukocytes to the *M. haemolytica* leukotoxin by increasing the expression of or activating the $\beta_2$ integrin CD11a/CD18 (LFA-1) on the leukocyte surface. *In vitro* exposure to proinflammatory cytokines (i.e. interleukin-1$\beta$, tumor necrosis factor-$\alpha$ and interferon-$\gamma$) increases LFA-1 expression on bovine leukocytes, which in turn correlates with increased binding and responsiveness to the leukotoxin. Alveolar macrophages and peripheral blood leukocytes from cattle with active bovine herpesvirus-1 (BVH-1) infection are more susceptible to the lethal effects of the leukotoxin *ex vivo* than leukocytes from uninfected cattle. Likewise, *in vitro* incubation of bovine leukocytes with bovine herpesvirus 1 (BHV-1) potentiates LFA-1 expression and makes the cells more responsive to leukotoxin. A striking characteristic of *H. somnus* infection is its propensity to cause vasculitis. We have shown that *H. somnus* and its lipo-oligosaccharide (LOS) trigger caspase activation and apoptosis in bovine endothelial cells *in vitro*. This effect is associated with the production of reactive oxygen and nitrogen intermediates, and is amplified in the presence of platelets. The adverse effects of *H. somnus* LOS are mediated in part by activation of endothelial cell purinergic receptors such as $P2X_7$. Further dissection of the pathways that lead to endothelial cell damage in response to *H. somnus* might help in the development of new preventive or therapeutic regimens. A more thorough understanding of *M. haemolytica* and *H. somnus* virulence factors and their interactions with the host might identify new targets for prevention of bovine respiratory disease.

**Keywords**: pathogenesis; infections; Mannheimia haemolytica; Haemophilus somnus

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Bovine respiratory disease (BRD) remains a significant economic problem for the beef and dairy cattle industries (Loneragan *et al.*, 2001). Despite the availability of a wide array of vaccines against the various agents involved in BRD, control and prevention remain a problem and a source of significant economic losses (Bowland and Shewen, 2000). In this report we present an overview of recent investigations from our group regarding the pathogenesis of two of the prominent bacterial agents of BRD: *Mannheimia haemolytica* and *Haemophilus somnus*. For the former, we focus on events related to the viral–bacterial synergism that contributes to the most severe manifestations of bovine pasteurellosis. For the latter, we will focus on recent investigations which demonstrate the ability of *H. somnus* to cause apoptosis in endothelial cells, and thus may contribute to the vasculitis that is commonly observed during *H. somnus* infections.

Bovine respiratory disease is a multifactorial disease problem. Environmental conditions, stressors and active infection with a number of respiratory viruses can predispose cattle to pneumonia caused by several bacterial pathogens. The most prominent among these is *M. haemolytica*, formerly known as *Pasteurella haemolytica*. This organism can cause a severe fibrinous pleuropneumonia ('shipping fever') in susceptible cattle. Both field evaluations and experimental studies have demonstrated that active viral infection with bovine herpesvirus type-1 or other respiratory viruses can predispose cattle to severe *M. haemolytica* pneumonia (Jericho and Langford, 1978; Babiuk *et al.*, 1996). The mechanisms responsible for the increased susceptibility are not clear. Although previous studies from several laboratories have indicated alterations in various leukocyte functions during viral infection (McGuire and Babiuk, 1984; Bielefeldt-Ohmann and Babiuk, 1985; Perler *et al.*, 2000), direct evidence linking these to the resulting susceptibility to *M. haemolytica* pneumonia is lacking.

*M. haemolytica* produces a variety of virulence determinants (Highlander, 2001). These include a capsule (Lo *et al.*, 2001), lipoproteins (Murphy *et al.*, 1998), iron binding proteins (Graham and Lo, 2002) an O-linked sialoglycoprotease (Abdullah *et al.*, 1992), various adhesins (Jaramillo *et al.*, 2000) and a leukotoxin (Shewen and Wilkie, 1982). More recently, a quorum sensing system was described for *M. haemolytica* (Malott and Lo, 2002) that might regulate the above virulence determinants in a coordinated manner, as has been described for *Bordetella pertussis* and other gram-negative pathogens. The most important virulence determinant of *M. haemolytica* is its leukotoxin, which is a 104-kDa protein that is secreted during the logarithmic growth phase. This leukotoxin is a member of the RTX family of toxins produced by a variety of gram-negative human and animal pathogens. Recent evidence indicates that the leukotoxin and other RTX toxins exert many of their biological effects via interactions with $\beta_2$ integrins on the surface of susceptible cells. The biological activity of the *M. haemolytica* leukotoxin involves interaction with bovine CD11a/CD18 (i.e. LFA-1) or CD18 alone (Li *et al.*, 1999; Jeyaseelan *et al.*, 2000; Deshpande *et al.*, 2002). There is a continuum of effects exhibited by bovine leukocytes exposed to leukotoxin. Small concentrations can activate leukocytes to undergo increased uptake of calcium and the release of reactive oxygen intermediates, eicosanoids and cytokines that can exacerbate local inflammation (Ortiz-Carranza and Czuprynski, 1992; Stevens and Czuprynski, 1995; Yoo *et al.*, 1995; Jeyaseelan *et al.*, 2001). If these processes also occur in the infected lung, they would help to explain the severe inflammation (fibrinous pleuropneumonia) that characterizes pasteurellosis. At greater leukotoxin concentrations impaired function of leukocytes is observed. Continued exposure, or high concentrations of leukotoxin, results in cell death by apoptosis or necrosis (Stevens and Czuprynski, 1996; Wang *et al.*, 1998; Cudd *et al.*, 2001; Deshpande *et al.*, 2002).

We became interested in investigating whether viral infection might influence expression or activation of $\beta_2$ integrins on the bovine leukocyte surface, and by so doing make these cells more susceptible to the adverse effects of the *M. haemolytica* leukotoxin. We first demonstrated that bovine neutrophils incubated *in vitro* with inflammatory cytokines (i.e. IL-l$\beta$, TNF-$\alpha$ or IFN-$\gamma$) exhibited increased LFA-1 expression and concomitant increases in leukotoxin binding and cytotoxicity (Leite *et al.*, 2002). We observed a significant correlation between neutrophil expression of LFA-1 (as determined by flow cytometry) and leukotoxin binding, cytotoxicity, apoptosis and caspase-3 activation (Leite *et al.*, 2002). Similar responses were observed for cytokine-treated peripheral blood mononuclear cells (PBMCs) subsequently exposed to leukotoxin (Leite *et al.*, 2004a,b). These observations are supported by the report of Lee *et al.* (2000), who provide *in vivo* evidence that $\beta_2$ integrins are important in the pulmonary inflammatory response to *M. haemolytica* infection in cattle.

We next examined whether active vine herpesvirus 1 (BHV-1) viral infection would affect the response of bovine lung and peripheral blood leukocytes to *M. haemolytica* leukotoxin *ex vivo* (Leite *et al.*, 2001). There was a substantial increase (nearly three-fold) in the numbers of bronchoalveolar lavage cells (principally mononuclear cells) recovered from the infected animals. This represents a substantial increase in the number of leukotoxin-responsive cells in the BHV-1 infected lung. We observed increased binding and cytotoxicity of leukotoxin for bronchoalveolar lavage cells at 4 days, and for peripheral blood leukocytes at 5 and 7 days, after viral infection. LFA-1 expression was enhanced at these time-points on the peripheral blood neutrophils, but not the BAL cells or the peripheral blood mononuclear cells from BHV-1 infected animals. However, we

cannot exclude the possibility that BHV-1 infection can activate $\beta_2$ integrins on BAL and PBMCs, without necessarily increasing the number of molecules on the leukocyte surface (Giancotti and Ruoslahti, 1999).

We next investigated whether *in vitro* exposure to BHV-1 would have a similar effect on bovine leukocytes. We noted increased expression of LFA-1 on peripheral blood mononuclear cells infected with BHV-1 *in vitro* for 24–72 hours. At the same time points, we also observed increased leukotoxin binding and cytotoxicity for the viral infected cells, as compared to control cells (Leite *et al.*, 2004a,b). Furthermore, the mononuclear cells produced increased IL-1β and IFN-γ mRNA in response to BHV-1 infection, as determined by real-time reverse transcription–polymerase chain reaction. Conditioned media from the virus-infected PBMCs enhanced expression of LFA-1 by naive neutrophils. The latter also exhibited increased binding of leukotoxin, and were more susceptible to killing by leukotoxin. These activities could be blocked by addition of a neutralizing anti-1L-1β antibody, indicating that 1L-1β is likely to be important in the leukocyte response to BHV-1 infection (Leite *et al.*, 2004a,b)

These data are consistent with our hypothesis that viral infection can lead to the release of mediators that enhance $\beta_2$ integrin expression on bovine leukocytes, and thus make the cells more responsive to the deleterious effects of the *M. haemolytica* leukotoxin. Figure 1 summarizes our observations and provides a working model to explain how viral infection might enhance the susceptibility of bovine cells to the *M. haemolytica* leukotoxin and by so doing contribute in part to the severe pulmonary inflammation that characterize bovine pasteurellosis.

These findings provide a new perspective for strategies to prevent or reduce the losses associated with pasteurellosis. It has been reported that prevention of viral infection by vaccination can reduce the severity of disease after subsequent *M. haemolytica* exposure (Jericho *et al.*, 1982). There are many vaccine products against *M. haemolytica* itself, most of which are bacterins or culture filtrates (Bowland and Shewen, 2000). Perhaps incorporation of new antigenic targets into conventional vaccines or the development of new modes of vaccine delivery (Lee *et al.*, 2001) will prove to be more efficacious in preventing losses associated with pasteurellosis. Other possibilities that might be considered include the use of agents that prevent the infiltration and activation of leukocytes in the lung. Several reports have suggested that administration of an L-selectin inhibitor can reduce the infiltration of granulocytes into the lung and the resulting lung damage during pasteurellosis (Radi *et al.*, 2001). Whether such molecules might be effective in preventing or reducing the severity of pasteurellosis in the field remains to be demonstrated. Likewise, it has been suggested that the effectiveness of tilmicosin in preventing pasteurellosis might result in part from its ability to reduce the number of neutrophils that can infiltrate the lung and respond to the leukotoxin (Chin *et al.*, 2000).

Our second area of interest is the pathogenesis of *Haemophilus somnus* infections. *H. somnus* causes a variety of disease syndromes, including respiratory disease, abortion, septicemia and thrombomeningoencephalitis (Humphrey and Stevens, 1983). Despite being somewhat fastidious in the laboratory, virulent strains of *H. somnus* are quite resistant to killing by serum (Corbeil *et al.*, 1985; Inzana *et al.*, 2002) and by

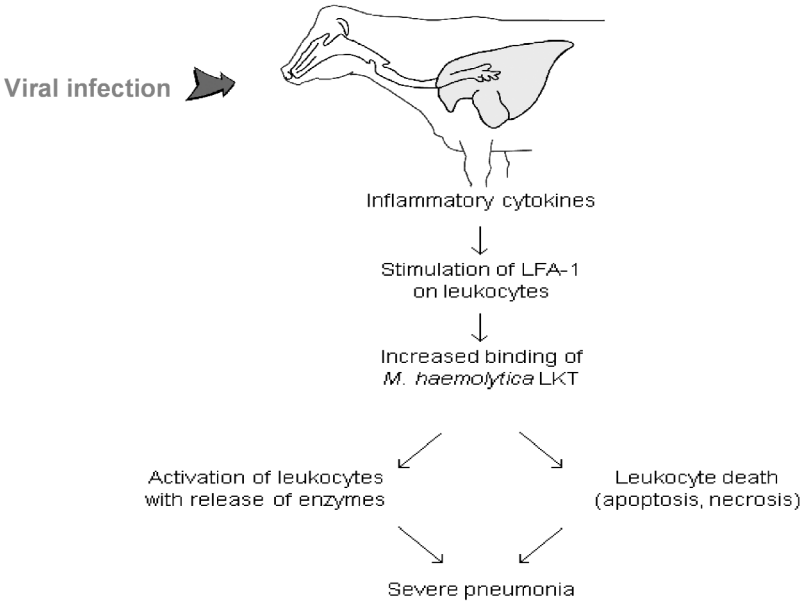


**Fig. 1** Working model of how viral infection might lead to increased susceptibility of bovine leukocytes to *M. haemolytica* leukotoxin (LKT).

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granulocytes and mononuclear phagocytes (Czuprynski and Hamilton, 1985; Lederer *et al.*, 1987; Gomis *et al.*, 1998). *H. somnus* has been reported to impair various activities of neutrophils and mononuclear cells and to multiply to some extent within the latter cells (Chiang *et al.*, 1986; Lederer *et al.*, 1987; Gomis *et al.*, 1997).

Vasculitis is a common occurrence during *H. somnus* infection in cattle and can be reproduced experimentally (Humphrey and Stevens, 1983; Gogolewski, *et al.*, 1987). The mechanism by which this occurs has not been elucidated. Earlier reports suggested that *H. somnus* can damage bovine endothelial cells *in vitro* (Kwiecien *et al.*, 1994). We were interested in determining whether *H. somnus* would cause apoptosis in bovine endothelial cells, which would perhaps explain the propensity of this agent to cause vascular lesions *in vivo*. In a previous report (Sylte *et al.*, 2001), we demonstrated that *H. somnus* and its lipo-oligosaccharide (LOS) cause apoptosis in bovine pulmonary artery endothelial cells *in vitro* in a dose- and time-dependent manner. Although close association of the bacteria with the surface of the endothelial cells can be observed, invasion is a rare event. This suggests that the organism exerts adverse effects on endothelial cells externally, via release of some component. The virulence determinants of *H. somnus* are not well understood and include LOS (Inzana *et al.*, 1997), several surface proteins (some of which have immunoglobulin-binding activity) (Corbeil *et al.*, 1997; Tagawa *et al.*, 2000), and both histamine (Ruby *et al.*, 2002) and nucleotides (Chiang *et al.*, 1986). We have focused on the effects of the *H. somnus* LOS, the best-characterized virulence determinant, on bovine endothelial cells, although it is possible that other virulence factors may also play a role. We have demonstrated that the LOS from several isolates of *H. somnus* causes chromatin condensation and caspase activation in bovine endothelial cells. This response is dependent on caspase 8 activation (Sylte *et al.,* 2003), although capase-9 activation could also be detected. Reactive oxygen and nitrogen intermediates are produced by the endothelial cells in response to the LOS (submitted for publication), which might cause oxidative stress to the cells, resulting in mitochondrial damage and cytochrome C release that could lead to caspase-9 activation. Although caspase-9 activation does not appear to be a primary initiation event in LOS-induced apoptosis, it may function to amplify the apoptotic signal.

We have focused considerable effort on the role of the P2X7 purinergic receptor in the response to LOS. This receptor forms ion-gated channels and has been demonstrated to have lipopolysaccharide-binding activity in other mammalian species (Ferrari and DiVirgilioi, 2000; Denlinger *et al.*, 2001). Furthermore, release of ATP or other nucleotides in response to LOS results in the activation of P2X7 and amplification of the effects of LOS on endothelial cells. We have demonstrated that adding the P2X7 antagonist o-ATP inhibits caspase-3 activation in bovine endothelial cells incubated with *H. somnus* LOS (M.J. Sylte, C.J. Kuckleburg, T.J. Inzana, P.J. Bertics and C.J. Czuprynski, submitted for publication). Blocking P2X7 with o-ATP significantly blocked LOS-induced caspase-8 activation, suggesting that P2X7 may, in part, be involved in transducing the apoptotic signal. More recently, we obtained preliminary evidence that *H. somnus* and its LOS can increase the expression ICAM-1 on bovine microvascular endothelial cells, and can activate bovine platelets (Kuckleburg *et al.*, 2004), as determined by increased Fas ligand (FasL) and CD40L expression. Activated platelets might release ATP and other nucleotides that could then stimulate the P2X7 receptor and have an adverse effect on endothelial cells. Addition of LOS-activated bovine platelets causes caspase activation and apoptosis in bovine endothelial cells *in vitro* (Kuckleburg *et al.*, 2004). This suggests that the activation of platelets by *H. somnus* or its LOS could result in damage to endothelial cells, which could in turn contribute to the vasculitis and thrombosis that are frequently observed during *H. somnus* infection *in vivo*. Figure 2 presents an overview of how *H. somnus* and its LOS might cause endothelial cell damage.

These observations suggest that the pathogenesis of the vascular lesions that occur during *H. somnus* infections may be quite complex. Efforts to protect against hemophilosis by vaccination need to take these points

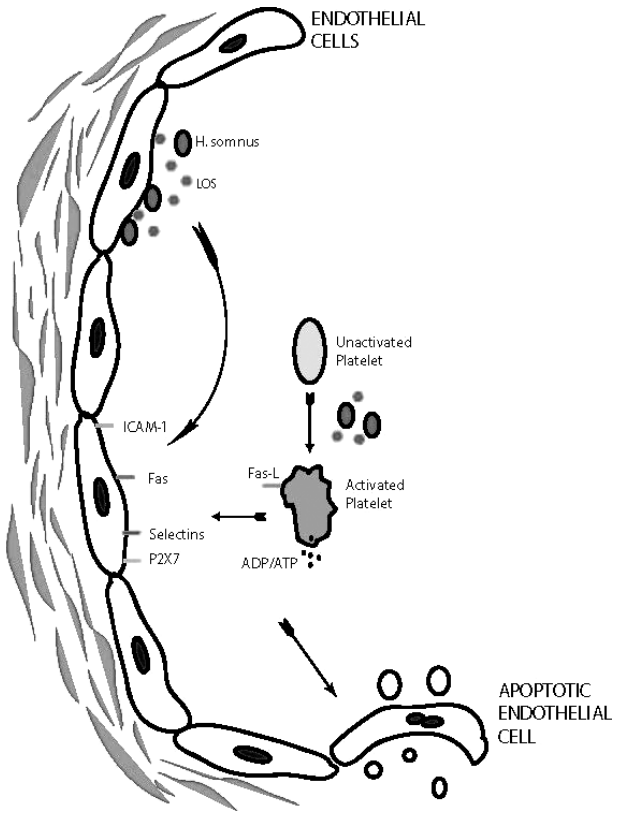


**Fig. 2** Working model of how H. somnus and its LOS might interact with platelets and endothelial cells to cause endothelial cell apoptosis.

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into consideration. All current vaccines are bacterins that are poorly defined. Because of the widespread carriage of *H. somnus* by healthy animals, elimination of infection is not a practical goal. Perhaps vaccines should focus on those aspects of the organism that lead to vascular lesions. Our observations provide targets for interrupting the interactions among LOS, endothelial cells and platelets that might prevent or reduce the vascular damage that occurs during *H. somnus* infection.

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