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Effect of experimental infection of cattle with bovine herpesvirus-1 (BHV-1) on the ex vivo interaction of bovine leukocytes with *Mannheimia* (*Pasteurella*) *haemolytica* leukotoxin

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Abstract

Mannheimia (*Pasteurella*) *haemolytica* A1 produces an extracellular leukotoxin (LKT) that is reported to bind the β_2-integrin CD11a/CD18 (LEA-1) on ruminant leukocytes. LKT binding induces activation, and subsequent cytolysis, of these cells. It is well known that active viral infection greatly increases the susceptibility of cattle to pasteurellosis. To better understand the mechanism by which this occurs, we investigated the effects of experimental in vivo infection of cattle with bovine herpes virus-1 (BHV-1) on the ex vivo interaction of bovine leukocytes with the *M. haemolytica* LKT. In this study, we demonstrated that active BHV-1 infection increased the expression of the β_2-integrin CD11a/CD18 (as defined by the mAb BAT75) on bovine peripheral blood neutrophils, enhanced the binding of LKT to bronchoalveolar lavage (BAL) leukocytes and peripheral blood neutrophils, and increased the killing of BAL leukocytes and peripheral blood leukocytes by LKT. In addition, BHV-1 greatly increased the number of BAL, resulting in many more LKT-responsive cells being present in the lungs. These findings might explain in part the increased susceptibility of BHV-1 infected cattle to pneumonic pasteurellosis.

Keywords: *Mannheimia haemolytica*; Pasteurellosis; BHV-1; Leukotoxin; Leukocytes; Bovine

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1. Introduction

The bovine respiratory disease (BRD) complex remains a major economic problem for both beef and dairy cattle industries in North America, and throughout the world (Frank, 1989; Wittum et al., 1996). One of the clinically severest components of BRD is pneumonic pasteurellosis (shipping fever), which is characterized by acute lobar fibronecrotizing pleuropneumonia with extensive polymorphonuclear leukocyte influx and death within pulmonary alveoli (Slocombe et al., 1985; Leite et al., 1999). *Mannheimia haemolytica* A1 is the primary bacterial pathogen responsible for shipping fever (Yates, 1982; Gonzalez and Maheswaran, 1993). *M. haemolytica* produces several virulence factors, foremost of which is a leukotoxin (LKT), whose cytotoxic activity is specific for ruminant leukocytes and platelets (Gonzalez and Maheswaran, 1993). The LKT is a Ca^{2+}-dependent pore-forming cytolysin, that is a member of the family of bacterial toxins designated RTX (repeats in toxin) (Welch, 1991). It has been reported that RTX toxins bind to β_2-integrins on target cells (Lally et al., 1997). *M. haemolytica* LKT was reported to bind to the β_2-integrin CD11a/CD18 (LFA-1), or CD18 alone, on bovine leukocytes (Ambagala et al., 1999; Li et al., 1999; Jeyaseelan et al., 1999; Leite et al., 2000). This binding induces formation of pore-like structures in the plasma membrane, resulting in both activation of leukocytes, and death by apoptosis and necrosis (Stevens and Czuprynski, 1995, 1996; Wang et al., 1998; Sun et al., 1999; Clinkenbeard et al., 1994).

Co-infection with a variety of respiratory viruses, including bovine herpesvirus-1, bovine virus diarrhea virus (BVDV), bovine parainfluenza-3, bovine respiratory syncytial virus and bovine coronavirus (Yates, 1982; Ohmann and Babiuk, 1985; Liu et al., 1999; Storz et al., 2000) increases the susceptibility of cattle to *M. haemolytica* LKT infection. The mechanism by which viral infection impairs resistance of cattle to pulmonary pasteurellosis has not been clearly elucidated. One mechanism that might be involved is the release of inflammatory cytokines during viral infection (Ohmann and Babiuk, 1985; Gonzalez and Maheswaran, 1993). These cytokines (i.e. IL-1β, TNF-α) can activate bovine leukocytes and modulate the migration, and functional activation of β_2-integrins on bovine leukocytes (Nagahata et al., 1995).

We hypothesized that BHV-1 infection might result in the release of IL-1β and other cytokines that enhance the expression of the putative LKT receptor CD11a/CD18 (i.e. LFA-1) on bovine leukocytes, thus amplifying their interaction with *M. haemolytica* LKT. The purpose of the present study was to determine whether BHV-1 infection increased CD11a/CD18 expression, LKT binding and LKT killing of bronchoalveolar lavage (BAL) and peripheral blood leukocytes in vitro. The results we obtained suggest that active BHV-1 infection enhances the biological response of bovine leukocytes to *M. haemolytica* LKT ex vivo.

2. Material and methods

2.1. Animals

Ten 12–14 month-old, crossbreed, beef calves (weight, 320 ± 20 kg) were used in this experiment. The 10 animals were maintained in a herd that was free of BHV-1 and BVDV. All animals in the herd were tested monthly for antibody to BVH-1 and BVDV to ensure

their viral-free status. One week before initiation of the experiment, all animals were tested and found to be free of detectable serum neutralizing antibodies to BHV-1. The animals were randomly assigned to two groups. Experimental infection of six calves was accomplished by intranasal inoculation of 10^6 plaque forming units (PFU) of BHV-1 (Cooper strain) suspended in 10 ml of RPMI 1640 (Mediatech, Cellgro, Herndon, VA). The four control animals were inoculated intranasally with 10 ml of RPMI 1640 alone.

2.2. Bronchoalveolar lavage

The animals were restrained in a chute and sedated with a combination of intravenous xylazine (0.03 mg/kg) and butorphanol (0.03 g/kg). The nostrils and external nares were wiped clean with sterile cotton gauze. BAL was then performed on each individual by holding the head and neck in extension and passing a 33 French semi-flexible tipped lavage catheter nasotracheally (Bivona Veterinary Products, Gary, IN). The catheter was wedged into a distal bronchus and held in place with an inflated cuff, after which approximately 120 ml of sterile physiologic saline was injected via the catheter. Rapid aspiration permitted harvest of greater than 60% of the injected fluid volume in each case. The lavage fluid was kept in an ice bucket until future assay were performed. At that time, the number of nucleated cells per milliliter was counted using a hemacytometer. Cytocentrifuge smears were prepared, stained with Diff-Quik, and examined microscopically to determine a differential cell count.

2.3. Peripheral blood leukocyte preparation

Briefly, peripheral blood was collected from the jugular vein of infected and control cattle using a 19 g needle attached to Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing sodium citrate (0.38% final volume) as anticoagulant. Blood was collected on days 0 (1 h before virus inoculation), 3, 5, and 7 post-infection. The blood was centrifuged ($250\times g$ for 20 min), and the platelet-rich plasma was removed. Leukocytes were obtained by rapid hypotonic lysis, washed twice in Hank's balance salt solution (HBSS), and resuspended at 10^6 cells/ml in RPMI 1640, with 2% fetal bovine serum. The leukocyte population was greater than 95% viable as estimated by Trypan blue exclusion.

2.4. Leukotoxin production and partial purification

A strain of *M. haemolytica* A1 (generously provided by Dr. R.E. Briggs, National Animal Disease Center USDA/ARS, Ames, IA) was used in this study as described previously (Tatum et al., 1998; Leite et al., 2000). Briefly, *M. haemolytica* A1 was inoculated onto blood agar and incubated overnight (Remel, Lenexa, KS) at 37 °C. The bacteria were washed from the agar surface with 10 ml of brain heart infusion broth that contained 0.5% yeast extract (BHI/YE; Difco, Detroit, MI) and incubated at 37 °C for 1 h while rotating (16 rpm) in 15 ml polypropylene tubes. A 10 ml aliquot of this suspension was then used to inoculate 200 ml of BHI/YE in a 500 ml Erlenmeyer flask. The flask was then incubated for 2 h at 37 °C with gentle shaking. The bacteria were collected by centrifugation ($1600\times g$ for 15 min), resuspended in 200 ml of RPMI 1640 supplemented with L-glutamine (4.0 mM), and incubated on a shaker apparatus for 4 h at 37 °C. The

bacteria were harvested by centrifugation ($1600 \times g$ for 20 min), and the crude leukotoxin-containing supernatant was collected and passed through a 0.45 μm pore size bottle top filter (Nalgen, Rochester, NY) to remove any residual bacteria. Aliquots (20 ml) of crude leukotoxin were concentrated over an Amicon ultra filtration unit equipped with a 62 mm diameter XM-50 ultra filtration membrane. The volume was then reduced to 10–20 ml over a 1–2 h period by applying 60 psi transmembrane pressure with nitrogen gas. The partially purified leukotoxin preparation that remained was collected and stored as 5 ml aliquots at −70 °C. Leukotoxic activity was confirmed by Trypan blue exclusion, using bovine peripheral blood leukocytes incubated with serial dilutions of toxin for 30 min at 37 °C. One unit of LKT activity was defined as the dilution causing 50% killing of bovine peripheral blood leukocytes as determined by Trypan blue exclusion. Partially purified LKT was stored at −70 °C until used in an experiment.

2.5. Biotinylation of LKT

Biotinylated LKT was prepared as described previously (Brown et al., 1997). Briefly, an 80:1 molar ratio of NHS-LC-biotin (Pierce Chemical, Rockford, IL) to partially purified LKT was incubated in an ice bath for 20 min. The mixture was then concentrated to 1 ml in a prechilled Amicon Centricon tube (50 kDa cutoff). The reaction was stopped by the addition of crystalline bovine serum albumin (30 mg), and incubated at 4 °C for 30 min. Unbound biotin was eliminated by buffer exchange over a Sephadex G-25 column ($1 \times 25\,cm^2$) using phosphate-buffered saline (PBS, pH 7.2) as elution buffer. The LKT eluted in the void volume in 7–8 min, as monitored by absorbance at 280 nm. Total protein in the eluted LKT was determined using the microplate Bradford assay. Leukotoxin activity was confirmed by incubating biotinylated LKT with bovine peripheral leukocytes for 30 min at 37 °C followed by Trypan blue exclusion.

2.6. LKT binding

Flow cytometry analysis of LKT binding was performed as described previously (Brown et al., 1997). Briefly, leukocytes (1×10^6) were incubated with biotinylated LKT (10 units) for 10 min at 4 °C. The cells were washed and resuspended in HBSS. Extra-avidin-fluorescein isothiocyanate (FITC) (4 μl) (Sigma, St. Louis, MO), was added, and the cells were incubated for 30 min at 4 °C. The cells were washed twice with HBSS and resuspended in 0.5 ml of HBSS. The stained cells were fixed with 0.4% paraformaldehyde (final concentration), washed and resuspended in HBSS. BAL and peripheral blood leukocytes were analyzed by flow cytometry with a Coulter Epics-Profile II flow cytometer (10,000 cells were scored for green fluorescence on a log scale). The peripheral blood leukocytes were gated into granulocyte (PMN) and mononuclear cell (PBMC) populations and analyzed by flow cytometry.

2.7. CD11a/CD18 mAb expression

Peripheral blood leukocytes and BAL from infected and control animals were incubated at 4 °C for 45 min, with an anti-CD11a/CD18 (BAT 75A). The BAT75 mAb is reported by

the supplier (VMRD, Pullman, WA) to bind both CD11a/CD18 (i.e. LFA-1), although the precise binding sites have not been reported. Following this, the cells were washed and resuspended in 50 μl of HBSS. A biotinylated $(Fab')_2$ rabbit anti-mouse IgG antibody (50 μg/ml, Sigma, St. Louis, MO) was added, and the cells incubated at 4 °C for 30 min. The cells were washed twice with HBSS, resuspended in 0.5 ml of HBSS, and fixed with 0.4% paraformaldehyde (final concentration). The cells were then washed and analyzed by flow cytometry with a Coulter Epics-Profile II flow cytometer (10,000 cells were scored for green fluorescence on a log scale). The peripheral blood leukocytes were gated into granulocyte (PMN) and mononuclear cell (PBMC) populations and analyzed by flow cytometry.

2.8. Cytotoxicity assay

Trypan blue exclusion was used to evaluate LKT cytotoxicity for bovine leukocytes. Because of the number of samples and time constraints, peripheral blood leukocytes were not separated into PMN and PBMC enriched populations. Briefly, BAL and peripheral blood leukocytes (1×10^6) were incubated with LKT (1 unit) for 1 h at 37 °C on a rotating platform. A 1:10 dilution of the cell suspension was prepared, an equal volume of 0.4% Trypan blue was added, and the mixture was incubated for 3 min at room temperature. Using a hemacytometer, at least 200 cells were counted and the percent cytotoxicity was calculated as $(\text{total number of dead (blue) cells}/\text{total number of cells}) \times 100$.

2.9. Statistical analysis

The flow cytometry data were analyzed using the WInMDI version 2.8 software. Other data were analyzed for statistical significance using a one-way analysis of variance followed by an unpaired *T*-test, using the Instat software program (GraphPad, San Diego, CA). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Total number of BAL cells are greatly increased in cattle experimentally infected with BHV-1

A 2.3-fold increase in cell numbers was observed $(P < 0.05)$ in BHV-1 infected cattle as compared with the control group on day 4 post-infection. The cell populations were primarily mononuclear cells; there was no difference in the number of PMNs between the groups (Fig. 1).

3.2. Effects of BHV-1 infection on CD11a/CD18 expression on bovine leukocytes

The expression of CD11a/CD18 on BAL (Fig. 2A), and PMNs and PBMCs from infected and control animals was examined by flow cytometry. Essentially all BAL, from both BHV-1 infected and control animals, stained positive for CD11a/CD18 (data not shown). There was no significant difference in mean channel fluorescence for CD11a/CD18 on BAL

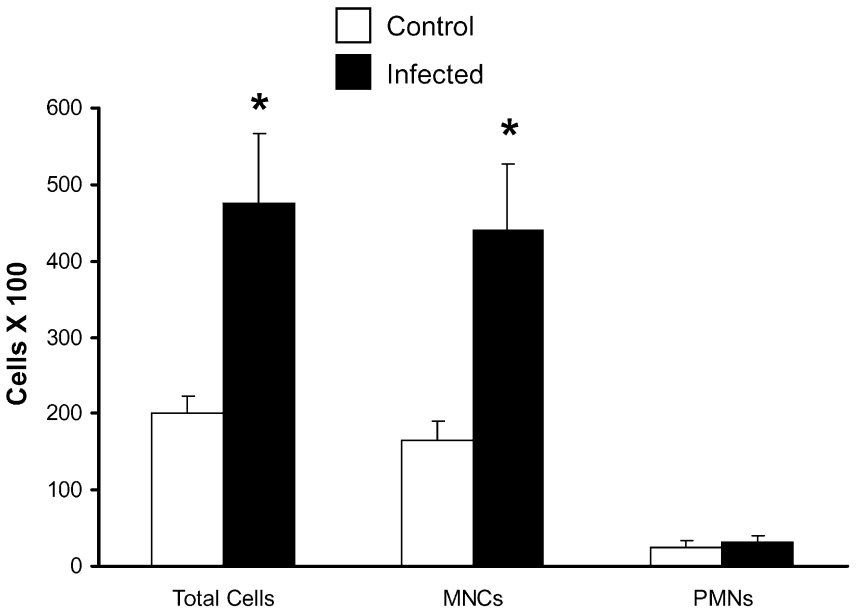

Fig. 1. Increased number of BAL cells recovered from BHV-1 infected cattle at day 4 post-infection. BAL cells were suspended in 1 ml of PBS, and counted using a hemacytometer. Cytocentrifuge smears were prepared, stained, and 100 cells from each sample were counted to determine the number of mononuclear and polymorphonuclear cells. The data represent the mean ± S.E. number of cells recovered from BHV-1 infected (six animals) and control cattle (four animals). Asterisks indicate statistically significant differences between BHV-1 infected and control animals ($P < 0.05$).

(day 4 post-infection) from BHV-1 infected and control cattle ($P > 0.05$) (Fig. 2A). We observed a slight increase in expression of CD11a/CD18 on days 3, 5 and 7 post-infection, however; these values were not different ($P > 0.05$) from control PBMCs (Fig. 2B). A significant increase ($P > 0.05$) in CD11a/CD18 expression was observed on PMNs at 5 and 7 days post-infection from BHV-1 infected animals, as compared to the PMNs from control animals (Fig. 2C).

3.3. BHV-1 infection enhances the binding of LKT to BAL and PBL

As illustrated in Fig. 3A, BAL from BHV-1 infected cattle exhibited a 1.9-fold increase in LKT-binding cells as compared with BAL from control cattle ($P < 0.05$). PBMCs from BHV-1 infected cattle exhibited a 1.4-fold ($P < 0.05$) increase in LKT-binding to the cells on day 5 post-infection (Fig. 3B). PMNs from infected cattle exhibited 1.8- and 2-fold increases ($P < 0.05$) in LKT binding on days 5 and 7 post-infection, respectively (Fig. 3C).

3.4. BHV-1 infection enhances the cytotoxicity of LKT for BAL and PBL

We observed an 11% increase in the cytotoxicity (based on Trypan blue exclusion) of LKT for BAL from BHV-1 infected cattle, compared with BAL from the control group,

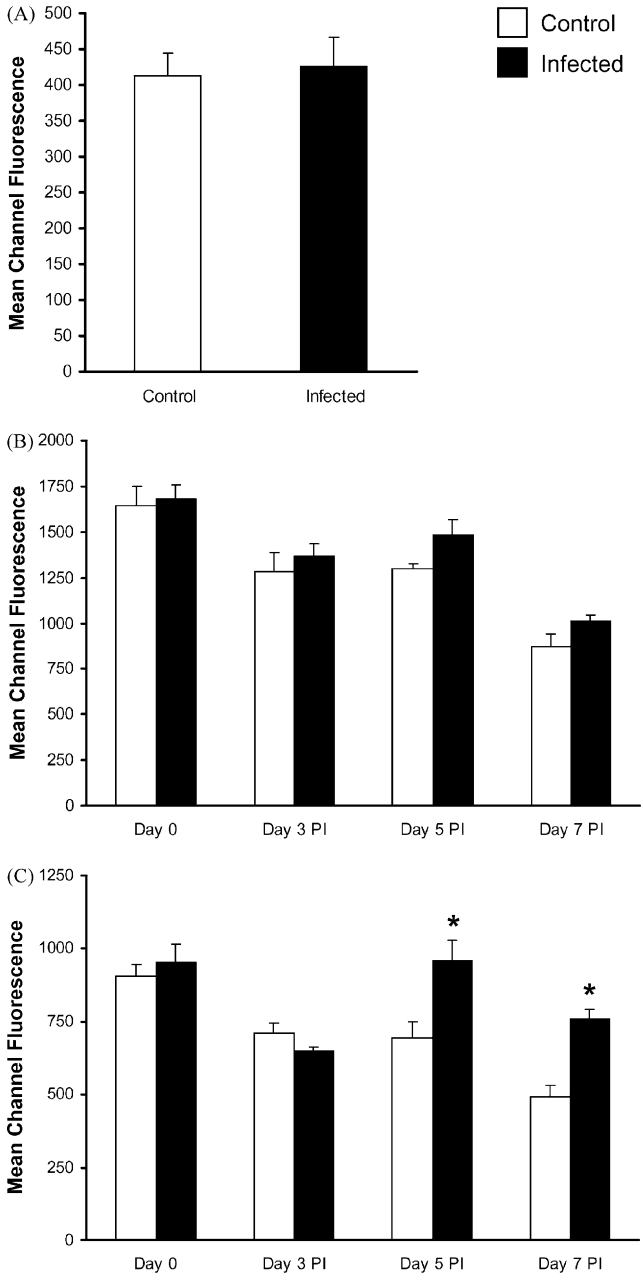

Fig. 2. CD11a/CD18 expression on BAL (A), PBMCs (B), and peripheral blood PMNs (C) from cattle at various times after experimental infection with BHV-1. Cell preparations from BHV-1 infected ($n = 6$) and control ($n = 4$) animals were incubated (40 min at 4 °C) with anti-CD11a/CD18a mAb antibody (BAT75A, 50 μg/ml final concentration). The cells were then washed, incubated with an FITC-labeled secondary antibody, and analyzed by flow cytometry (10,000 cells were scored for green fluorescence). The data represent the mean ± S.E. channel fluorescence of BHV-1 infected (six animals) and control animals (four animals). Asterisks indicate statistically significant differences between BHV-1 infected and control animals ($P < 0.05$).

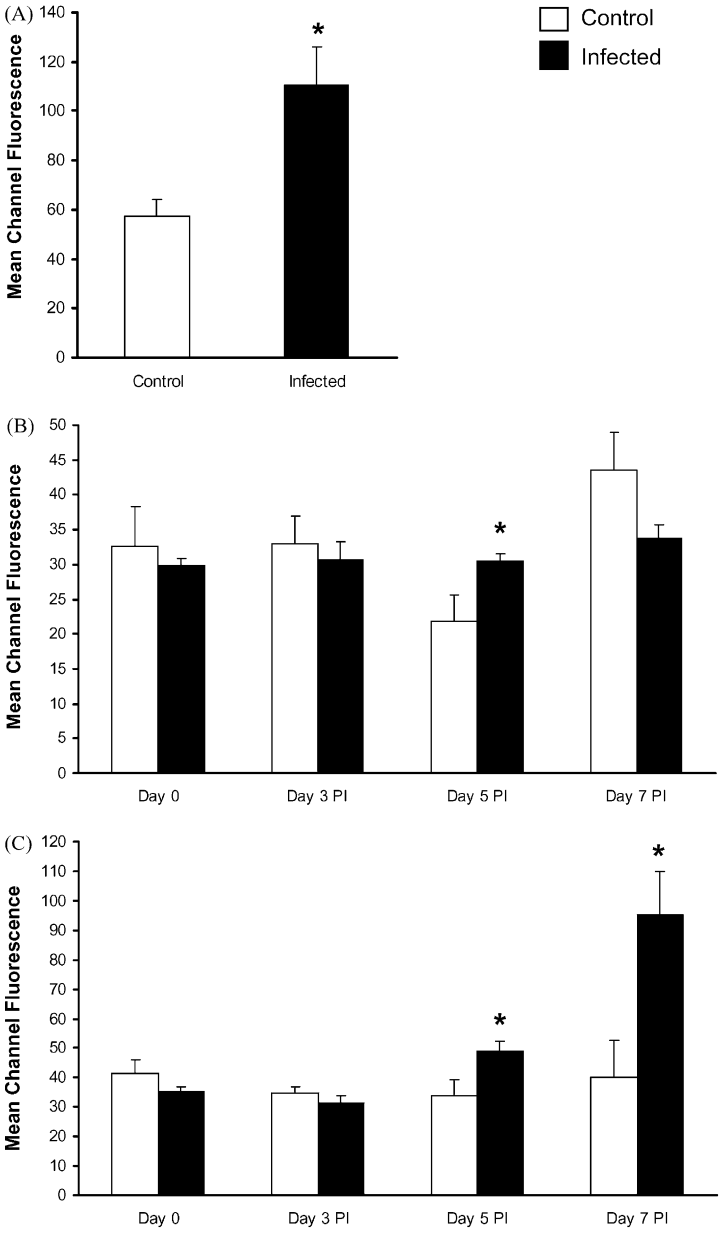

Fig. 3. *M. haemolytica* LKT binding to BAL (A), PBMCs (B), and peripheral blood PMNs (C) from cattle experimentally infected with BHV-1. Cells from infected and control animals were incubated with biotinylated *M. haemolytica* LKT (10 units) for 10 min on ice. The cells were washed, extra-avidin-FITC was added and the cells incubated on ice for 20 min. The stained cells were washed, fixed with 4% paraformaldehyde, and analyzed by flow cytometry (10,000 cells were scored for green fluorescence). The data represent the mean ± S.E. channel fluorescence of cells from BHV-1 infected (six animals) and control animals (four cattle). Asterisks indicate statistically significant differences between BHV-1 infected and control animals ($P < 0.05$).

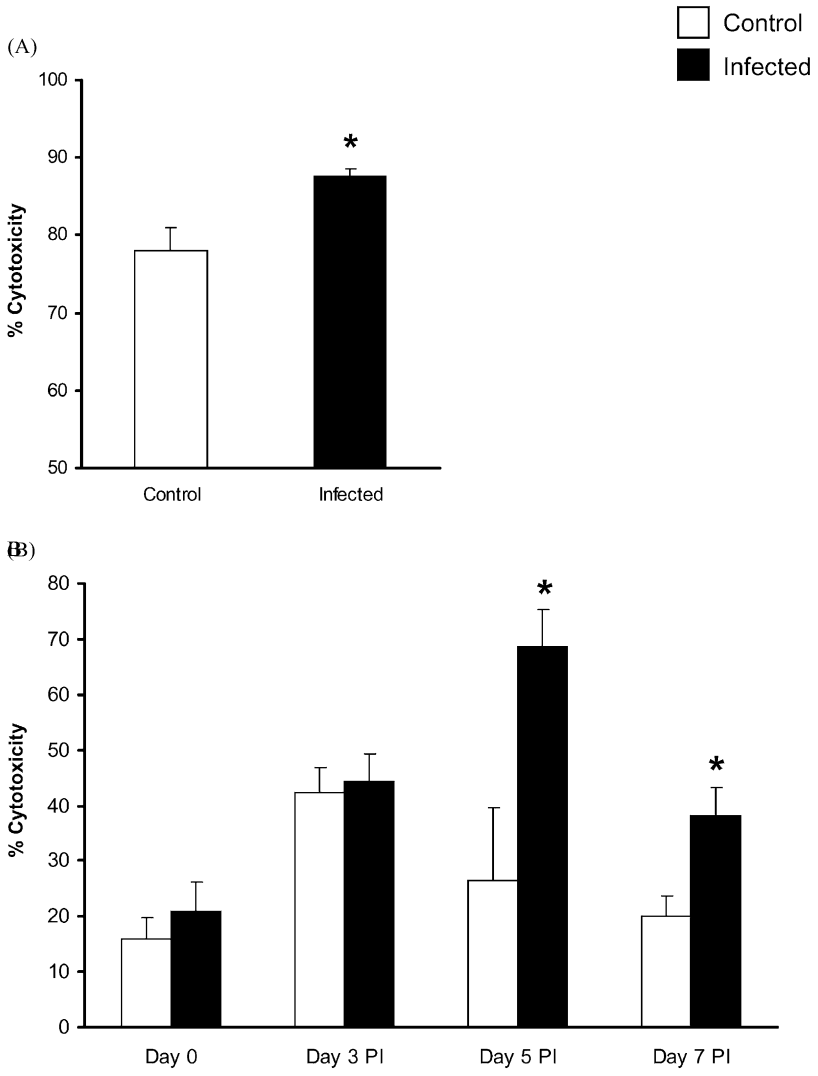

Fig. 4. *M. haemolytica* LKT cytotoxicity for BAL (A), and PBL (B) from cattle experimentally infected with BHV-1. Cells were incubated with *M. haemolytica* LKT (10 units) for 1 h at 37 °C, and cell viability assessed by Trypan blue exclusion. The data represents the mean ± S.E.M. percent of cytotoxicity for cells from BHV-1 infected ($n = 6$) and control animals ($n = 4$). Asterisks indicate statistically significant differences between BHV-1 infected and control animals ($P < 0.05$).

at day 4 post-infection (Fig. 4A). Because of the number of samples and assays being performed, we did not separate peripheral blood leukocytes into PMN and PBMC enriched populations. We observed a 42 and 18% increase in the cytotoxicity of LKT for PBL from BHV-1 infected cattle at days 5 and 7, respectively, as compared with peripheral blood leukocytes from control cattle (Fig. 4B).

4. Discussion

In this study, we demonstrated that BHV-1 infection increases the ex vivo interaction of *M. haemolytica* leukotoxin with bovine leukocytes. We recovered greatly increased number of BAL from the BHV-1 infected cattle, with alveolar macrophages and other mononuclear cells being the most prominent cell populations as reported previously (Foreman et al., 1981). The presence of more LKT-responsive cells in the lungs of BHV-1 infected cattle could play an important role in the pathogenesis of pasteurellosis. LKT-mediated activation and death of increased number of BAL would corroborate with the intense lung inflammation and damage noted in pasteurellosis (Slocombe et al., 1985; Wittum et al., 1996). It is presumed that the inflammatory process elicited by BHV-1 infection results in secretion of cytokines locally and into the systemic circulation (Ohmann and Babiuk, 1985; Yoo et al., 1995). These inflammatory cytokines would induce increased expression of adhesion molecules on leukocytes and endothelial cells resulting in increased migration and functional activation of β_2-integrins on leukocytes in the lung (Arnaout, 1990; Williams and Solomkin, 1999).

In this study, we observed increased cytotoxicity of LKT for PBLs at 5 and 7 days post-infection. Peripheral blood mononuclear cells exhibited a slight increase in LKT binding at day 5 post-infection ($P > 0.05$), whereas PMNs exhibited increased CD11a/CD18 expression and increased LKT binding at 5 and 7 days post-infection ($P < 0.05$). These observations support the previously reported association between LFA-1 expression, and the binding and cytotoxic activity of LKT for bovine peripheral blood leukocytes (Ambagala et al., 1999; Li et al., 1999; Jeyaseelan et al., 1999; Leite et al., 2000). We believe it is likely that the increased CD11a/CD18 expression by PMNs from BHV-1 infected cattle is mediated by inflammatory cytokines secreted during viral infection, rather than the direct effects of the virus. This supposition is based on reports that productive infection by BHV-1 does not occur in bovine peripheral blood leukocytes (Foreman et al., 1982a,b). We have previously reported that bovine PMNs stimulated with IL-1β in vitro increased CD11a/CD18 expression, LKT binding and cytotoxicity (Leite et al., 2000). These latter effects were inhibited by addition of the same BAT75 anti-CD11a/CD18 mAb used in the present study. The BAT75 mAb is reported to bind to both CD11a and CD18, although the precise binding site has not been determined. As a result, our results do not allow us to exclude the possible contributions of other β_2-integrins (i.e. CD11b/CD18) in our flow cytometry studies. Recently, we have obtained preliminary data indicating that incubation of bovine PMNs with other cytokines (TNF-α, IFN-γ) also upregulates CD11a/CD18, and LKT binding and killing (unpublished observations).

Bovine lung leukocytes constitutively expressed β_2-integrins; the mAb (BAT75A) used in this study recognized nearly all BAL cells from both BHV-1 infected and uninfected cattle (Leite et al., 2000). Nor did we observe differences in the intensity of CD11a/CD18 expression by BAL cells from BHV-1 infected or control cattle (Fig. 2A). In contrast, we observed a significant increase in LKT binding (Fig. 3), and LKT mediated killing (Fig. 4), of BAL from BHV-1 infected cattle. Stimulation of leukocytes can lead to conformational changes in the β_2-integrin molecules that result in increased receptor affinity, rather than increased receptor number (Hibbs et al., 1991; Barnett et al., 1998). Thus, one possible explanation for the apparent discrepancy between increased LKT binding to BAL, without

increased CD11a/CD18 expression, is that the inflammatory process in the BHV-1 infected lung stimulated a conformational change in CD11a/CD18 that increased its affinity for LKT. We have previously reported that RGD peptides block the binding of LKT to resting, but not IL-1β stimulated, bovine PMNs (Leite et al., 2000). This prior observation is also consistent with the inference that CD11a/CD18 on stimulated bovine leukocytes has an increased affinity for LKT. However, we cannot have the possibility that the mAb (BAT75A) recognize other β_2-integrins on leukocytes that do not play a role as a receptor to LKT, or that BAL cells possess an additional LKT receptor, besides CD11a/CD18.

There are some limitations in our study. First, we used only one mAb (BAT75A) to evaluate CD11a/CD18 expression. The binding site for this mAb has not been fully characterized. Thus, it is possible it might bind to other members of the β_2-integrin family. A second limitation is that we did not distinguish which peripheral blood cell population was most susceptible to the action of LKT, since we did not separate these cells into PMN and PBMC enriched populations before assessing cytotoxicity. Although the method used to assess cytotoxicity (Trypan blue exclusion) is tedious and more subjective than some assays, it has been used by our laboratory and others to determine cell viability, and offers relatively rapid results as compared to other methods (i.e. color development in MTT or XTT assays). One final limitation of our study was the day that we chose to perform the BAL (day 4 post-infection). This time was chosen because some previous reports suggested that it fell within the window of increased susceptibility to pasteurellosis (Yates, 1982).

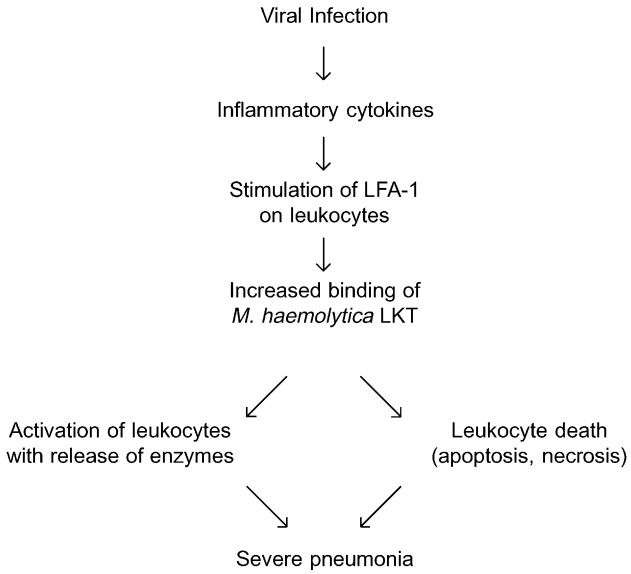

Fig. 5. Proposed model for the mechanism of viral–bacterial synergism in BRD. Viral infection induces the release of inflammatory cytokines that stimulate CD11a/CD18 expression on leukocytes. This will enhance *M. haemolytica* LKT binding to bovine leukocytes. LKT binding will activate leukocytes to release inflammatory mediators (e.g. reactive oxygen intermediates, enzymes, etc.) that will damage lung tissue. Eventually, the LKT kills the leukocytes and impairs the clearance of bacteria from the lungs. Together, these effects will contribute to the severe pneumonia that characterizes BRD.

However, we observed an increase in CD11a/CD18 expression and LKT binding to PMNs on day 5 and 7 post-infection, suggesting that, ideally, BAL would be performed more than once after infection (i.e. 3–7 days post-infection). However, we were concerned that performing BAL more than once on the same animal would confound our analyses. Financial constraints prevented us from adding more animals that would have allowed us to sample BAL, at more than one time point, without lavaging any animal more than once.

Based on our observations, we suggested that one possible explanation for the viral–bacterial synergism observed in BRD is that BHV-1 infection results in the release of cytokines, which in turn attract lung leukocytes, and increase leukocyte expression of CD11a/CD18. When *M. haemolytica* infects the lower airways of a BHV-1 infected cow, it finds a favorable environment with increased number of lung leukocytes. These may have activated CD11a/CD18, which facilitates LKT binding and killing of these cells.

Once established in the lungs, the continued release of LKT and other bacterial virulence factors (i.e. LPS) by *M. haemolytica* would stimulate the continued release of inflammatory cytokines (Yoo et al., 1995; Morsey et al., 1999) that would sustain CD11a/CD18 expression and exacerbate the effects of *M. haemolytica* LKT. Based on our observations, we have devised a working model for the mechanism by which inflammatory cytokine stimulation of LFA-1 on leukocytes of BHV-1 infected cattle would predispose cattle to pasteurellosis (Fig. 5).

Acknowledgements

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