Feedlot Session

Moderator: Tom Noffsinger

Abrupt Weaning Significantly Increases Mortality Following a Secondary Bacterial Respiratory Infection

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Abstract

Secondary bacterial respiratory infections are a major cause of mortality in fall-weaned feedlot calves, and epidemiological studies implicate a variety of stressors as significant contributing factors. Experimental studies have identified stressors that may compromise pulmonary defense mechanisms but there is no evidence that these functional changes alter respiratory disease outcome. We used a model of combined viral and bacterial respiratory disease to determine if nutritional and psychological stressors (abrupt weaning; AW) altered respiratory disease mortality. Mortality was doubled in AW calves challenged with *Mannheimia haemolytica* four days after a primary bovine herpesvirus-1 (BHV-1) respiratory infection, when compared to calves adapted to weaning (pre-conditioned; PC) for two weeks prior to respiratory challenge. Reduced survival time and decreased lung pathology in the AW group suggested death was due to an acute systemic reaction. Viral shedding did not differ significantly between the two treatment groups. AW calves and all calves developing fatal pneumonia had significantly elevated interferon (IFN)-γ levels in nasal secretions and increased systemic pro-inflammatory responses. Analysis of blood leukocytes revealed significantly increased CD14 and TLR4 gene expression in animals with fatal pneumonia. These analyses support the conclusion that stress enhanced innate immune responses to viral infection without altering the level of BHV-1 infection. These studies provide the first quantitative evidence that stress associated with abrupt weaning contributes significantly to fatal bovine respiratory disease.

Keywords: abrupt weaning, BHV-1, interferon, *Mannheimia haemolytica*, stress, viral-bacterial synergy

Résumé

Les infections respiratoires secondaires causées par les bactéries sont une cause majeure de mortalité chez les veaux de parcs d'engraissement sevrés à l'automne. À cet égard, les études épidémiologiques ont identifié une variété de facteurs de stress qui favorisent ces infections de manière significative. Des expériences ont montré que certains facteurs de stress peuvent compromettre les mécanismes de la défense pulmonaire, mais on n'a pas de preuve que ces modifications fonctionnelles influencent l'apparition d'une maladie respiratoire. Nous avons utilisé un modèle combinant des infections respiratoires virale et bactériologique pour déterminer si des facteurs de stress nutritionnels et psychologiques (sevrage brusque, SB) altéraient la mortalité due aux maladies respiratoires. Nos résultats montrent que la mortalité a doublé chez les veaux sevrés brusquement (SB) et exposés à la bactérie Mannheimia haemolytica quatre jours après une infection respiratoire primaire à l'herpèsvirus-1 bovin (HVB-1), par rapport à des veaux adaptés au sevrage (pré-conditionnés, PC) pendant deux semaines avant l'exposition respiratoire. Une diminution du temps de survie et du degré de pathologie des poumons dans le groupe des veaux SB suggère que la mort fut causée par une réaction systémique aiguë. L'excrétion du virus n'a pas varié significativement entre les deux groupes de traitements. Les veaux SB et tous les veaux qui sont morts d'une pneumonie avaient un taux d'interférons (IFN)-γ significativement plus élevé dans leurs sécrétions nasales et une réponse pro-inflammatoire systémique significativement plus prononcée. L'analyse des leucocytes sanguins a révélé une expression significativement plus importante des gènes des molécules CD14 et TLR4 chez les veaux morts de pneumonie. Ces analyses corroborent l'hypothèse que le

stress stimulerait la réaction immunitaire naturelle à l'infection virale sans modifier le niveau d'infection par l'HVB-1. Mais surtout, cette étude apporte la première preuve quantitative que le stress provenant du sevrage brusque contribue de manière significative à la mort des bovins des suites d'une maladie respiratoire.

Introduction

A fatal synergy exists between the combined response to a primary viral and a secondary bacterial respiratory infection. This viral-bacterial synergy has been associated with an increased incidence and severity of secondary bacterial respiratory infections in cattle and can be replicated experimentally.[4,5,48] Several potential mechanisms have been identified by which a primary viral respiratory infection may increase susceptibility to secondary bacterial infections. Increased bacterial attachment and invasion are important contributing factors, but there is also evidence that viral-induced leukocyte recruitment to the lung and the production of pro-inflammatory cytokines contributes to lethal viral-bacterial synergy.[6] Thus, any factor that alters recruitment and activation of pulmonary leukocytes during a primary viral infection may impact the severity of a secondary bacterial respiratory infection.

Studies in mice have demonstrated that specific psychological stressors may either enhance[39] or inhibit[16,27,38] lung leukocyte recruitment and cytokine secretion following a primary influenza infection. Furthermore, an experimental influenza infection study in humans revealed an association between psychological stress and increased production of IL-6, a pro-inflammatory cytokine.[15] Thus, psychological stressors can significantly modulate host responses to primary viral respiratory infections. No studies have been performed, however, to investigate the effects of either psychological or physical stressors on viral-bacterial synergy and the outcome of a secondary bacterial respiratory infection.

Epidemiological studies have implicated a variety of stressors, including transportation, weaning, social reorganization, and dietary changes, with an increased incidence and severity of bovine respiratory infections.[28,29] The bovine respiratory disease complex is often complicated by the occurrence of a primary viral infection followed by a secondary bacterial infection. Several viral and bacterial pathogens have been implicated in the viral-bacterial synergy of fatal bovine respiratory disease (BRD), but bovine herpesvirus-1 (BHV-1) and the gram-negative bacteria, *Mannheimia haemolytica* are two important pathogens for which reproducible experimental infection models have been developed.[3,49] BHV-1 infection is usually not fatal, but aerosol infection with *M. haemolytica* four days after BHV-1 infection of naïve calves results in 30-70% mortality.[3]

This model of BRD has been used extensively to identify immune mechanisms which might contribute to the viral-bacterial synergy observed following a primary BHV-1 infection.[5,7,8,9] These mechanisms include altered alveolar macrophage function, altered polymorphonuclear leukocyte (PMN) function, decreased NK-cell activity, and increased production of pro-inflammatory cytokines. The increased production of pro-inflammatory cytokines that occurs during a primary BHV-1 infection is of particular interest in view of the pathology associated with an acute *M. haemolytica* respiratory infection.[1] Within hours of bacterial colonization of the lung there is a necrotizing inflammatory response that is dependent upon PMN recruitment to the lung[40] and increased production of pro-inflammatory cytokines such as IL-1, IL-8, and TNF-α.[34,50] The bacterial components that contribute to the activation of these inflammatory responses include capsule polysaccharide, lipopolysaccharide (LPS), and leukotoxin.[11,47] Although many of the host responses that occur during BHV-1 and *M. haemolytica* respiratory infections have been characterized, this model has not been used to critically address the question whether stress significantly alters the viral-bacterial synergy that causes a fatal respiratory infection.[18]

We hypothesized, based on evidence from mouse models of stress and influenza infection,[16,27,38,39] that stress-induced changes in immune responses during a primary BHV-1 infection would significantly alter mortality following a secondary *M. haemolytica* infection. The stressors in our model included social reorganization and nutritional changes associated with abrupt separation of suckling calves from their mothers[25] and subsequent transportation. These management practices are similar to those previously associated with an increased incidence of BRD.[28,29] A variety of clinical, immunological, and molecular analyses were then performed to identify possible mechanisms by which stress may alter fatal viral-bacterial synergy. Understanding these mechanisms has important implications for effectively managing or treating bovine respiratory disease.

Materials and Methods

Treatment groups

Female and castrated male, 5 to 6-month-old, crossbred (Angus X Hereford) calves were selected from the same herd. Calves seronegative for BHV-1 and *M. haemolytica* were randomly assigned to one of two groups (n = 10 per group). One group (pre-conditioned; PC) was separated from their dams and adapted to a ration of hay and oats for two weeks prior to transport to the Vaccine and Infectious Disease Organization (VIDO) Animal Facility and initiation of the BRD challenge. The second group of calves (abrupt weaned; AW) remained on pasture and suckled their dams until their separation immediately

prior to transport. These calves were deemed to have undergone psychological and nutritional stress. The day prior to BHV-1 challenge, calves from both experimental groups were transported together for 3.5 hours then housed in a single pen at the VIDO Animal Facility. Blood samples to isolate serum, peripheral blood mononuclear cells (PBMC), and PMN were collected from the jugular vein prior to challenge with BHV-1 and daily thereafter until death. Experiments were conducted according to the *Guide to the Care and Use of Experimental Animals*, provided by the Canadian Council on Animal Care. All experimental protocols were approved by University of Saskatchewan Animal Care Committee.

Experimental infection

All calves were aerosol challenged with BHV-1 isolate 108 ($5x10^7$ pfu/animal) the day following transport. Four days later, all animals were challenged with an aerosol of *M. haemolytica* strain PH45 ($6x10^9$ cfu/animal). Aerosol challenges were performed using an Ultra-Neb 99 nebulizer.[a] The strain and dose of viral and bacterial challenge and the interval between primary viral infection and secondary bacterial challenge were previously optimized to ensure clinical signs of respiratory disease in all calves with a mortality rate between 30-70%.[3] Shedding of infectious BHV-1 in nasal secretions was monitored daily by collecting nasal mucus, beginning on the day of viral challenge. Briefly, sterile cotton swabs were used to collect nasal mucus and then immersed in 1 mL minimum essential medium[b] prior to being frozen at -112°F (-80°C) until tested. Virus in nasal secretions was quantified by plaque titration in microtiter plates with a neutralizing antibody overlay as previously described.[42] Following *M. haemolytica* challenge, the animals were monitored every three hours for disease status. Animals that were unable to rise from recumbency were euthanized by intravenous injection of 100 mg sodium pentobarbital/kg body weight[c]. The presence of bacterial infection in animals with fatal respiratory disease was confirmed by culture from lung swabs collected during postmortem examination.

Gross pathology

Pathological scoring of the entire lung was performed to quantify grossly visible lung lesions known to be present during *M. haemolytica* colonization and characterized by tissue consolidation, congestion, and a fibrinous pleuropneumonia.[1] Scoring was performed by a clinical veterinarian blinded to treatment groups. Each lobe was visually examined with the lung positioned dorsal side down and palpated before estimating the percentage of each lobe that was affected. Lung lobes were given a value representing that lobe's percentage of total lung volume (Value A). These values are as follows: right cranial lobe = 6%; right posterior cranial lobe = 5%; right middle lobe = 7%; right caudal lobe = 35%; right intermediate lobe = 4%; left cranial lobe = 5%; left posterior cranial lobe = 6%; and left caudal lobe = 32%. The percentage of each lobe affected (Value B) was multiplied by the percentage volume for that lobe (Value A) and the sum of these products (A x B) was recorded as a total lung score (out of 100%) for each animal.

Clinical responses

Rectal temperature, body weight, and nasal lesions were monitored daily by a clinical veterinarian blinded to treatment groups. Serum and blood leukocytes were collected daily until death or five days following *M. haemolytica* infection. The experiment was terminated on the fifth day following bacterial challenge and all surviving animals were treated with 10 mg tilmicosin/kg body weight.[d] Nasal secretions for the analysis of IFN-γ and IFN-α were collected from all animals prior to BHV-1 challenge, at three days post-BHV-1 challenge, and at six days post-BHV-1 challenge. Nasal secretions were collected by inserting a cotton tampon into one nostril for 20 minutes and absorbed fluid was expressed from the tampon by compressing it in a 50 mL plastic syringe. Nasal secretions were stored at -112°F (-80°C) until IFN levels were analyzed by ELISA.

ELISAs

Calves were screened for serum antibodies specific for BHV-1 and *M. haemolytica* using ELISA. Detection of BHV glycoprotein D (gD)-specific antibodies was performed as previously described.[10] Titers below 1/40 dilution of serum were considered negative. Serum antibodies specific for *M. haemolytica* leukotoxin were detected as previously described and titers below 1/400 dilution of serum were considered negative.[26] The level of serum haptoglobin, a marker of acute inflammatory responses during bovine respiratory disease, was determined using a bovine specific ELISA.[22] The concentration of IFN-γ and IFN-α in nasal secretions was determined by capture ELISA as previously described.[36]

Cell isolation

Blood was collected from the jugular vein into Vacutainers®[e] containing K_3 EDTA 7.5% TriPotassium solution and PBMC and PMN leukocytes were isolated as described previously.[46]

Gene expression analysis

Total RNA was isolated from aliquots of 10×10^6 PBMC or PMN leukocytes using TRIZOL Reagent[f] and further purified using RNeasy Mini-columns[g] as described previously.[2] RNA concentration was determined with an Agilent 2100 Bioanalyzer using RNA 6000 Nano kit.[h] cDNA was prepared by reverse transcription of 500ng total PBMC RNA or 100ng of total PMN RNA using the

SuperScript™ III Platinum® Two-Step qRT-PCR Kit with SYBR® Green[i] following the manufacturer's protocol. For each PCR reaction, 5 ng of cDNA was amplified with each primer set using the following parameters: 122°F (50°C) for two minutes to eliminate carry-over dUTP, then 45 cycles of 203°F (95°C) for 15s; 131°F (55°C) for 30s; and 161.6°F (72°C) for 30s. Detection of bovine TLR4, IL-1β and IFN-γ used oligonucleotide primers described elsewhere.[23,30,31] Primers for bovine CD14 were designed from accession NM_174008 and are as follows: sense (5'-CACCACCCTCAGTCTCCGTAAC) and antisense (5'- GCGAGTGTGCTTGGGCAATG). Primers for bovine IL-10 were designed from accession NM_174088 and are as follows: sense (5'-GCTGTATCCACTTGCCAACC) and antisense (5'-CCAGGTAACCCTTAAAGTCATCC). Primers for bovine 2'5' oligoadenylate synthetase (2'5' OAS) were designed from accession NM_178108 and are as follows: sense (5'-GTGCGAGAACCAGAGGAGAG) and antisense (5'-TATTCTTATGCTTCATCTTACACAGTTG). Primers for bovine TNF-α were designed from accession NM_173966 and are as follows: sense (5'-GTAGCCGACATCAACTCTC) and antisense (5'-AGGACCTGTGAGTAGATGAG). Primers for bovine GAPDH were designed from accession AJ000039 and are as follows: sense (5'- GGCAAGTTCAACGGCACAGTCAAG) and antisense (5'- GTGCAGGAGGCATTGCTGACAATC). All primers were designed using Clone Manager 7[j] and designed to span introns where possible. Samples were amplified in duplicate using a Bio-Rad *iCycler* and a melt curve was completed following each PCR reaction to ensure that fluorescence quantification was specific to the PCR amplified product. PCR products from custom designed primers were validated by sequence analysis using a Beckman CEQ2000XL. Amplification data are expressed as change in Cycle threshold (ΔCt) calculated as follows: (ΔCt = Cycle threshold of Gene of Interest – Cycle threshold of GAPDH). A smaller ΔCt equates to more abundant gene expression.

Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 3.03 software.[k] Differences in mortality were analyzed using a chi-square test to compare survival curves. Linear regression analyses were performed to determine if a significant correlation existed between lung damage versus day of death and secretion of IFN-α versus IFN-γ. A two-tailed Student's t-test assuming unequal variances was used for analyses of differences (days to death, daily body temperatures, serum haptoglobin, IFN-γ secretion, and gene expression) between treatment groups or when animals were grouped by disease outcome. One way analyses of variance (ANOVA) were used with Tukeys post-test when comparing changes in values over time (serum haptoglobin, gene expression) within treatment groups.

Results

The effect of abrupt weaning on a secondary bacterial respiratory infection

Although stress has been linked with an increased incidence and severity of viral respiratory infections in both humans[14,15] and mice,[39] the impact of stress on a secondary bacterial respiratory infection has not previously been evaluated. To address this issue, a group of calves that were nutritionally and psychologically stressed by abrupt separation from their dams and transportation immediately prior to a primary BHV-1 respiratory infection (AW group) were compared to a group of pre-conditioned animals that had been adapted to the dietary change and social reorganization associated with maternal separation (PC group). Clinical disease and mortality were then monitored following a secondary bacterial challenge with *M. haemolytica*. The AW group had twice the mortality following a secondary bacterial infection than the PC group (Figure 1A). Mortality in the stressed group was characterized by a significantly shorter survival time when compared to the PC group ($P < 0.007$) (Figure 1B) and there was a direct correlation ($r^2 = 0.53$; $P < 0.01$) between increased lung pathology and survival time (Figure 1C). These observations suggested the rapid onset of mortality in the stressed group was not directly due to increased bacterial infection and lung pathology.

Pro-inflammatory responses following primary viral infection

Since pro-inflammatory responses play an important role in the pathogenesis of *M. haemolytica* infection,[11,47] we hypothesized differences in the response of AW calves to BHV-1 infection contributed to fatal respiratory disease. The production of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α can induce fever and the synthesis of acute-phase proteins such as haptoglobin.[22] To determine if stress altered the acute-phase responses to BHV-1 infection, we monitored body temperature and serum haptoglobin levels in both treatment groups. In agreement with Babiuk *et al*[3] BHV-1 infection induced fever in all infected animals, but this increase in body temperature on day 4 post-BHV-1 infection was significantly greater for the AW group than the PC group ($P < 0.05$) (Figure 2A). Furthermore, when compared to pre-infection levels only the AW calves had significantly elevated serum haptoglobin on day 4 post-BHV-1 infection ($P < 0.005$) (Figure 2B). These observations support the conclusion that stress associated with abrupt weaning significantly altered host responses to the primary viral respiratory infection.

Calves in both AW and PC groups developed fatal secondary bacterial infection. Increased mortality in the AW group suggested that stress may either induce a nov-

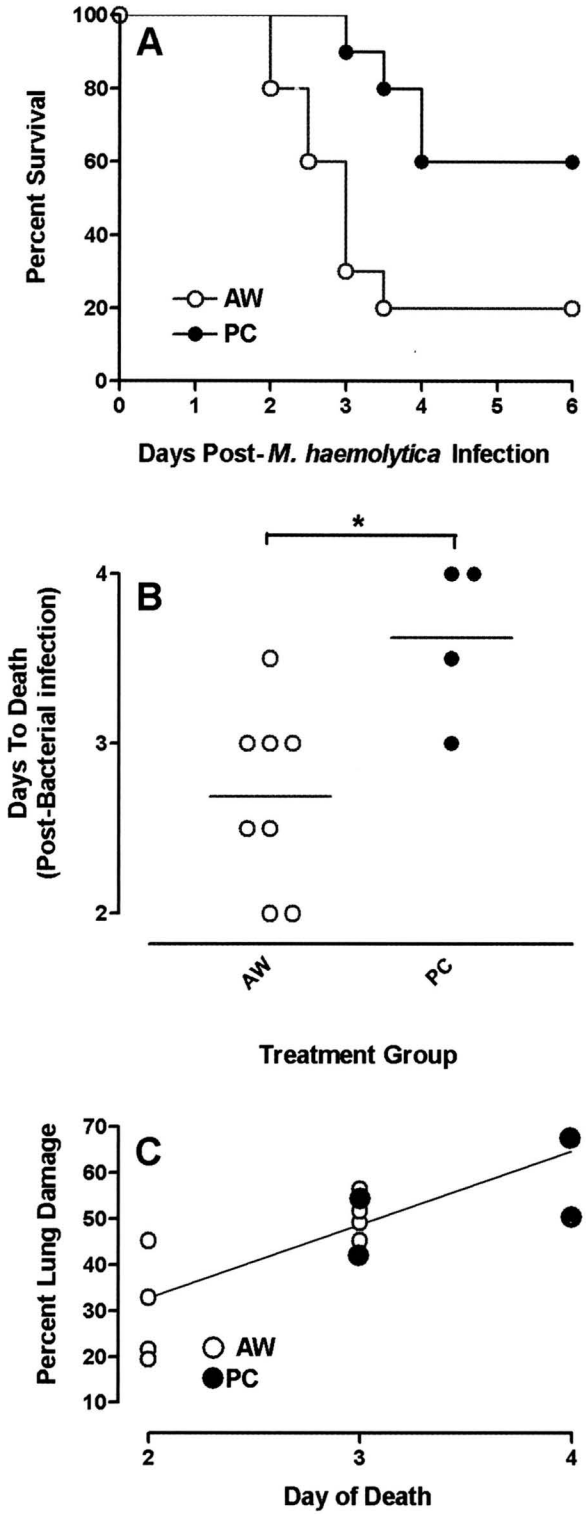

Figure 1. Abrupt weaning increases mortality and reduces survival time following a secondary bacterial respiratory infection. Animals were monitored every three hours for clinical signs of disease following aerosol challenge with *M. haemolytica* as described in Materials and Methods. (A) Percent survival of either abruptly weaned (AW; open circles) or pre-conditioned (PC; closed circles) treatment groups. (B) Comparison of time interval to death for AW and PC groups after aerosol challenge with *M. haemolytica*. Values are presented for individual animals and the horizontal bar represents median value for each group. $^*P < 0.05$. (C) There was a significant correlation between survival time following bacterial challenge and visible lung pathology ($r^2 = 0.53$, $P < 0.01$).

el mechanism of viral-bacterial synergy or significantly enhance viral-induced responses which usually contribute to a fatal secondary bacterial respiratory infection. Therefore, we re-analyzed the clinical responses to BHV-1 infection on the basis of BRD outcome rather than by stress treatment groups. These analyses revealed there was no significant difference in fever responses when comparing animals that died (n = 12) versus survivors (n = 8) (data not shown). Furthermore, weight loss as a measure of anorexia following BHV-1 infection[3] was not significantly different when comparing survivors versus fatal bacterial infection (data not shown). There was, however, a significant ($P = 0.01$) elevation in serum haptoglobin on the day of *M. haemolytica* challenge among animals that died versus survivors (Figure 2C). The results of these analyses provided evidence that the magnitude of the acute-phase response following a primary viral respiratory infection may be linked to the severity of a secondary bacterial respiratory infection.

Antiviral responses following BHV-1 infection

Psychological stress can significantly enhance cytokine responses to influenza infections in mice[39] and humans,[15] and stress can alter antiviral responses due to glucocorticoid modulation of cytokine production[12] and viral replication.[44] Therefore, we analyzed BHV-1 replication and antiviral responses in both treatment groups. The level of viral shedding in nasal secretions was monitored, but no significant differences in BHV-1 replication were observed between treatment groups (Figure 3A) or when animals were grouped by disease outcome (data not shown). BHV-1 is a potent inducer of both IFN-α and IFN-γ, and peak IFN levels in nasal secretions are usually associated with peak viral titers.[7] These cytokines can also induce fever[8] and elevate serum haptoglobin levels.[20] Therefore, to determine whether the stress of AW significantly altered anti-viral responses we analyzed IFN levels in nasal secretions. Despite no differences in viral shedding, IFN-γ levels in nasal secretions were significantly ($P < 0.05$) greater in the AW than the PC calves on day 3 post-BHV-1 infection (Figure 3B). There was, however, no significant difference in IFN-γ levels when comparing treatment groups after the secondary bacterial challenge. Although there was a significant correlation ($r^2 = 0.63$; $P < 0.01$) between IFN-α and IFN-γ concentrations in nasal secretions of individual animals, there was no significant difference in IFN-α secretion when treatment groups were compared (data not shown). Therefore, the correlation between BRD outcome and host response to a viral infection was analyzed for IFN-γ alone (Figure 3C). Grouping animals by disease outcome revealed a significant increase ($P < 0.05$) in the antiviral response for animals that died when compared to survivors. Thus, stress associated with abrupt weaning enhanced both the acute-phase and antiviral responses during the primary viral infection and the increased amplitude in these responses correlated significantly with a fatal secondary bacterial respiratory infection.

Altered capacity to respond to gram-negative bacteria precedes fatal pneumonia

Bacterial capsular polysaccharide, LPS, and leukotoxin have been implicated in the pathogenesis of fatal *M. haemolytica*-induced pneumonia, and LPS has been linked to the induction of pro-inflammatory cytokines.[11,47] Cytokine responses to LPS are known to be mediated primarily through the mammalian toll-like receptor (TLR) 4–MD2–CD14 signaling complex (TLR4 complex)[32] and IFN-γ enhances expression of the TLR4 complex.[33,41] To determine if TLR4 complex expression was one mechanism by which stress might enhance fatal viral-bacterial synergy, we analyzed CD14 and TLR4 gene expression in PBMC and PMN of all calves. Quantitative RT-PCR analyses revealed a similar trend of increased expression for both CD14 (Figure 4A) and TLR4 (Figure 4C) in PBMC of all calves following BHV-1 infection. In contrast, striking differences in the transcriptional levels of CD14 (Figure 4B; $P < 0.005$) and TLR4 (Figure 4D; $P < 0.001$) were observed when animals were segregated according to disease outcome. This comparison revealed that animals with fatal BRD consistently had greater CD14 and TLR4 expression levels at the time of bacterial challenge. Perhaps more importantly, only dead animals displayed significantly ($P < 0.005$) increased TLR4 and CD14 expression when comparing day 4 post-BHV-1 infection with pre-infection levels on day 0 (Table 2). Finally, significant changes in TLR4 and CD14 gene expression levels were not observed in PMN (data not shown), which may be important since PMNs are the major cell population recruited to the lung after *M. haemolytica* infection. The qRT-PCR data support the conclusion that BHV-1 infection increases the capacity of PBMC to respond to LPS.

Viral-induced expression of pro-inflammatory cytokines

Pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α, have been implicated as important factors contributing to clinical responses and viral-bacterial synergy following BHV-1 infection.[9] Differences in AW and PC calves' clinical responses (Figure 2) prompted us to analyze expression of pro-inflammatory cytokines during BHV-1 infection and determine if a correlation existed between cytokine expression and fatal BRD. IL-1 expression in PBMC did not change significantly throughout the course of BHV-1 infection (Table 1 and 2) and IL-6 expression remained below or at the threshold of detection (Ct > 35) prior to bacterial challenge (data not shown). In contrast, TNF-α expression in PBMC was

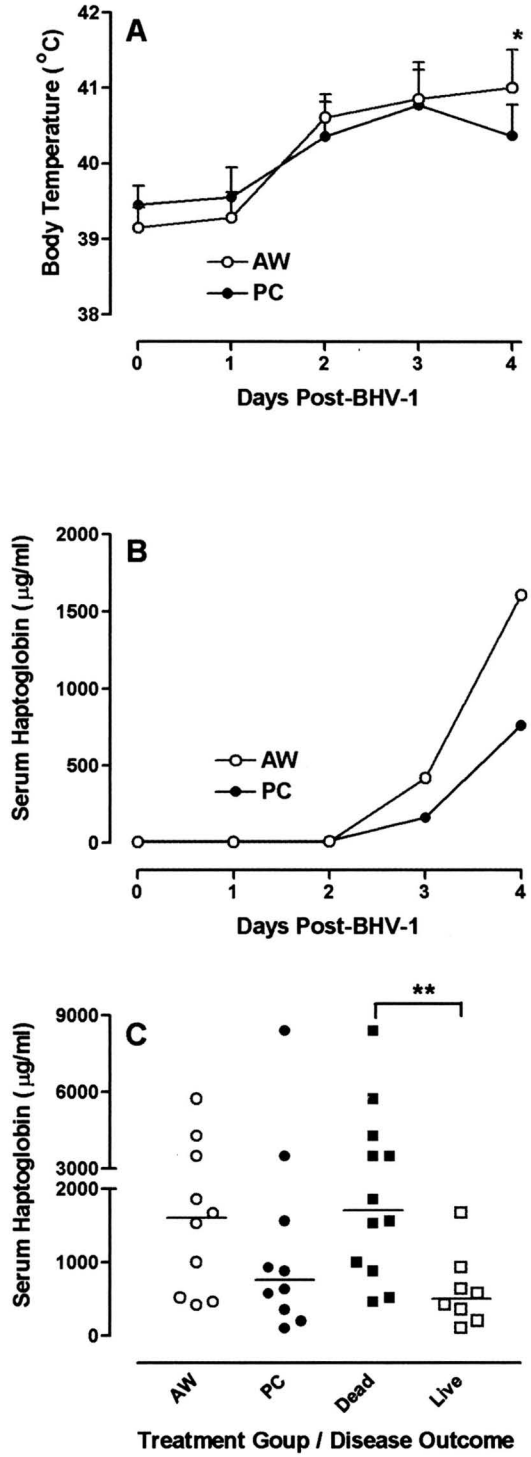

Figure 2. Acute-phase response following BHV-1 infection. (A) Rectal temperature of animals presented as mean + 1SD of values for abrupt weaned (AW; open circles) or pre-conditioned (PC; closed circles) groups. Values were compared between groups for each day post BHV-1 infection. *$P < 0.05$. (B) Serum haptoglobin levels were analyzed daily following BHV-1 infection and data presented are median values for the AW and PC treatment groups. Haptoglobin levels in the AW group were significantly ($P < 0.01$) elevated on day 4 when compared to pre-infection levels (day 0). (C) Serum haptoglobin levels were quantified immediately prior to *M. haemolytica* challenge. Data presented are values for individual animals and data are presented for the AW and PC groups (n = 10/group) and sorted for disease outcome following *M. haemolytica* challenge. Animals that died (Dead; n = 12) or survived (Live; n = 8) following bacterial infection include calves from both treatment groups. **$P = 0.01$.

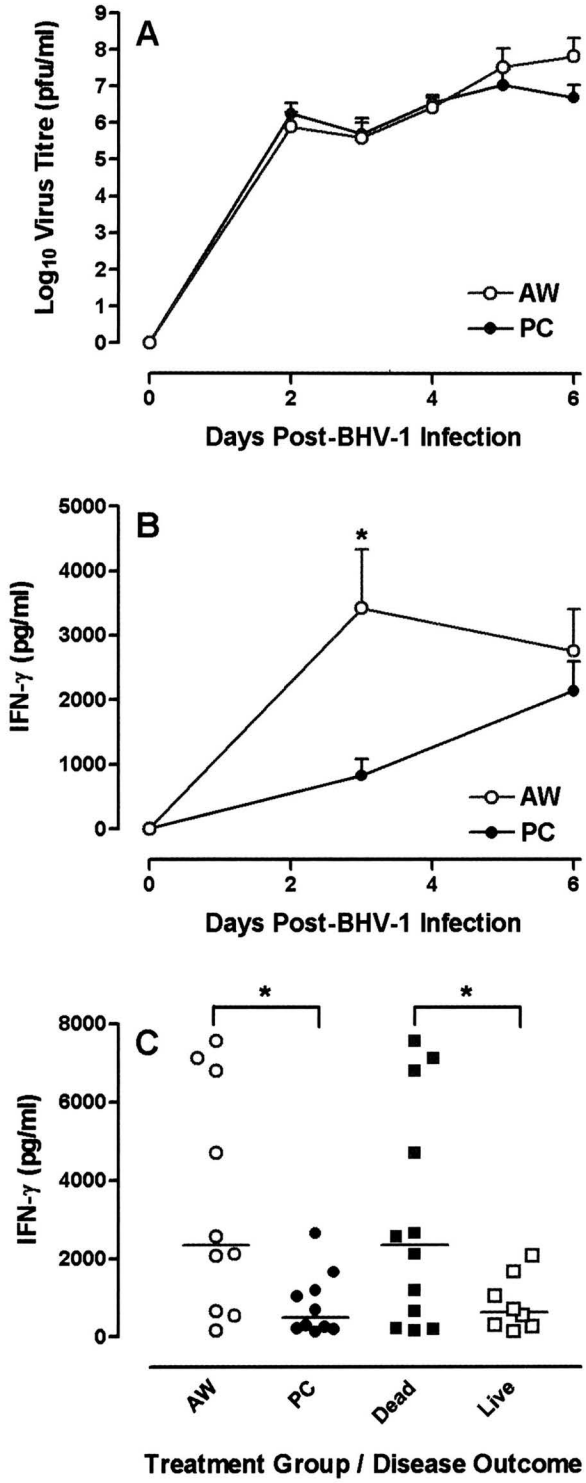

Figure 3. Stress alters antiviral responses, but not viral replication. (A) Infectious BHV-1 shed in nasal secretions. Data presented are the mean + 1SD of values for the abrupt weaned (AW; open circles) or pre-conditioned (PC; closed circles) groups. (B) IFN-γ concentration in nasal secretion was determined by capture ELISA. IFN-γ secretion was compared between the AW group (open bar) and PC group (closed bar) for day 3 and day 6 post-BHV-1 infection. Data presented are the mean + 1SD of values for the AW and PC groups. *$P < 0.05$. (C) IFN-γ levels on day 3 post-BHV-1 with values presented for individual animals. Data presented are for the AW and PC groups (n = 10/group) and sorted for disease outcome following *M. haemolytica* challenge. Animals that died (Dead; n = 12) or survived (Live; n = 8) following bacterial infection include calves from both the AW and PC groups.*$P < 0.05$.

significantly elevated following BHV-1 infection in both treatment groups (Table 1), but did not correlate with disease outcome (Table 2).

IL-10 expression also increases during an inflammatory response, but the kinetics of IL-10 expression may be important for negative regulation of pro-inflammatory cytokine production.[19,37] IL-10 expression in PBMC was found to be significantly ($P < 0.005$) elevated in both treatment groups prior to *M. haemolytica* infection, but this increase was significantly ($p < 0.05$) greater for AW versus PC calves (Table 1). Furthermore, the increase in IL-10 expression was significantly greater in animals that developed fatal BRD ($P < 0.005$) (Table 2). Thus, no significant correlation was observed between pro-in-

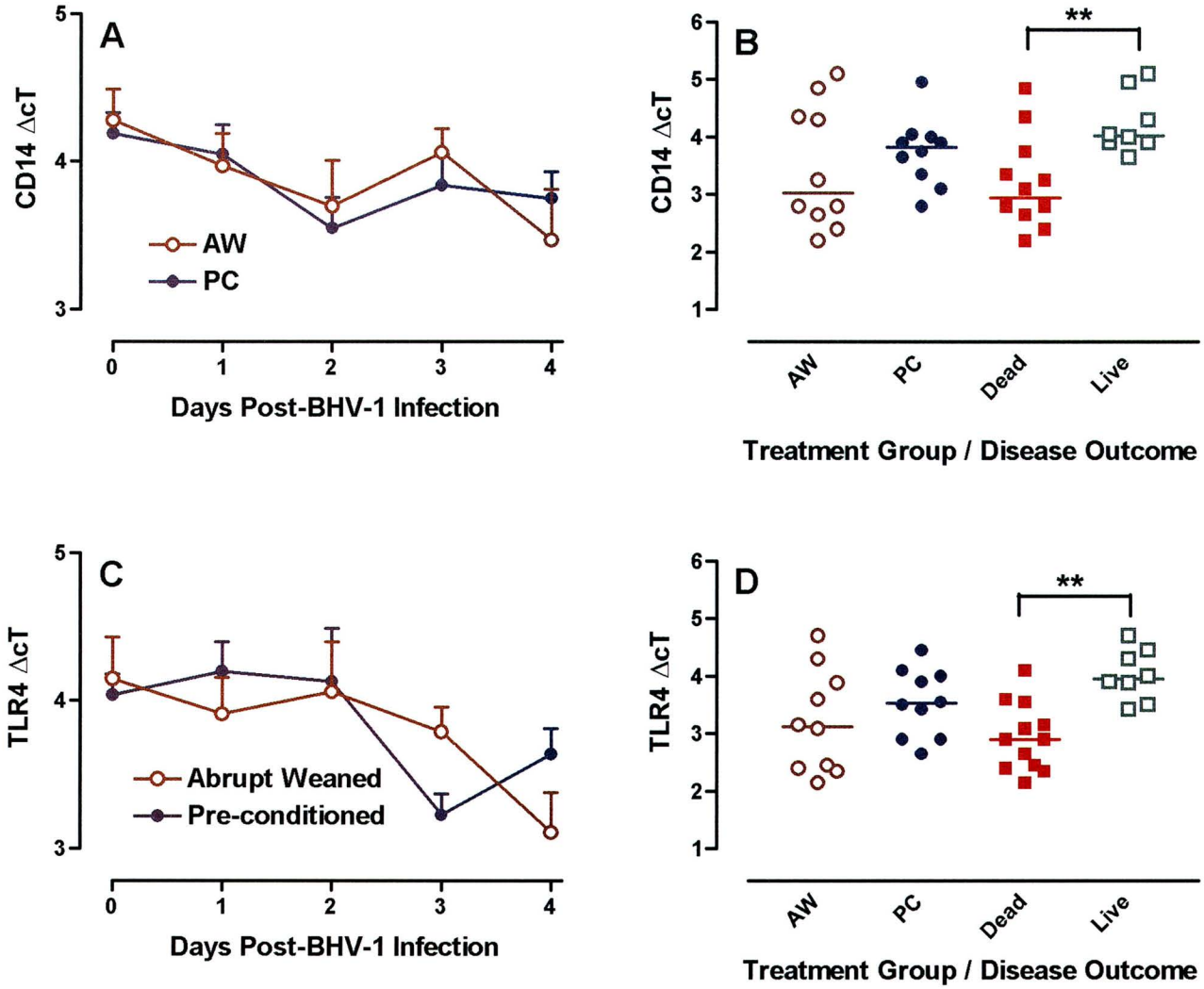

Figure 4. CD14 and TLR4 expression in PBMC changes following BHV-1 infection. (A) qRT-PCR analysis of CD14 expression levels (ΔCt) in PBMC during BHV-1 infection. Data presented as mean + 1SD of values for the abrupt weaned (AW; open circles) or pre-conditioned (PC; closed circles) groups. A smaller ΔCt corresponds to greater mRNA expression (B) CD14 gene expression levels on day 4 post-BHV-1 with data presented as ΔCt values for individual animals. Data presented are for the AW and PC groups (n = 10/group) and sorted for disease outcome following *M. haemolytica* challenge. Animals that died (Dead; n = 12) or survived (Live; n = 8) bacterial infection include calves from both the AW and PC groups. $^{**}P < 0.0005$. (C) qRT-PCR analysis of TLR4 expression levels (ΔCt) in PBMC following BHV-1 infection. Data presented as mean + 1SD of values for the AW and PC groups. (D) TLR4 expression levels on day 4 post-BHV-1 with data presented as values for individual animals. Data presented are for the AW and PC groups (n = 10/group) and sorted for disease outcome following *M. haemolytica* challenge. Animals that died (Dead; n = 12) or survived (Live; n = 8) following bacterial infection include calves from both the AW and PC groups. $^{**}P < 0.0005$.

flammatory cytokine (IL-1, IL-6, and TNF-α) transcription during viral infection or with fatal viral-bacterial synergy. In contrast, a significant correlation between IL-10 expression and fatal BRD supports the hypothesis that dysregulation of pro-inflammatory responses may contribute to fatal viral-bacterial synergy.

IL-10 can induce IFN-γ secretion by NK cells.[45] Therefore, elevated IFN-γ levels in nasal secretions of animals with fatal BRD (Figure 3C) may have been induced by increased IL-10 expression. Quantitative RT-PCR analysis of IFN-γ expression in PBMC revealed that IFN-γ expression did not correlate with IL-10 and, in fact, remained constant in both treatment groups following BHV-1 infection (Table 1). In contrast, expression of 2'5' OAS, an interferon-induced gene, was markedly up-regulated in all animals following viral infection (Table 1). In addition, expression of 2'5' OAS expression increased significantly ($p < 0.005$) in animals with fatal BRD but not in survivors (Table 2). Although there was no correlation between IL-10 and IFN-γ gene expression during BHV-1 infection, the increased expression of 2'5' OAS in PBMC is consistent with the elevated IFN-γ levels measured in nasal secretions (Figure 3C). Therefore, increased IFN-γ levels in nasal secretions may reflect a local recruitment or activation of NK cells rather than a systemic response to viral infection. Elevated 2'5' OAS

Table 1. Effect of stress on gene expression levels during BHV-1 infection.

	Abrupt weaned[A]		Pre-conditioned[A]	
Genes	Day 0[B]	Day 4[C]	Day 0	Day 4
2'5' OAS	3.3 (± 2.8) [D]	0.9 (± 2.2) *	2.7 (± 2.1)	0.0 (± 1.5) **
IFN-γ	10.1 (± 0.8)	10.1 (± 0.8)	10.0 (± 0.8)	10.3 (± 1.0)
IL-1β	6.8 (± 0.9)	6.7 (± 0.8)	7.0 (± 0.8)	6.7 (± 0.6)
TNF-α	6.1 (± 0.4)	4.8 (± 0.7) **	6.0 (± 0.4)	4.9 (± 0.3) **
IL10	8.2 (± 0.8)	5.7 (± 0.6) ** §	8.1 (± 0.6)	6.1 (± 0.3) ** §
CD14	4.3 (± 0.7)	3.5 (± 1.1)	4.2 (± 0.4)	3.7 (± 0.6)
TLR	44.1 (± 0.9)	3.2 (± 0.9) *	4.0 (± 0.4)	3.5 (± 0.6) *

[A]Animals treated a described in M&M; n = 10 per group.
[B]Data from PBMC collected prior to BHV-1 infection.
[C]Data from PBMC just prior to *M. haemolytica* challenge.
[D]Data are represented as mean ΔCt (± SD). A smaller ΔCt equates to more abundant gene expression.
* = $P < 0.05$ vs day 0.
** = $P < 0.005$ vs day 0.
§ = $P < 0.05$ vs other group's day 4.

Table 2. Effect of disease outcome on gene expression levels during BHV-1 infection.

	Animals that die[A]		Animals that survive[A]	
Genes	Day 0[B]	Day 4[C]	Day 0	Day 4
2'5' OAS	2.5 (± 2.1) [D]	0.0 (± 1.2) **	3.6 (± 2.9)	1.2 (± 2.5)
IFN-γ	9.9 (± 0.7)	10.3 (± 0.8)	10.2 (± 0.8)	10.0 (± 1.0)
IL-1β	6.9 (± 0.8)	6.6 (± 0.8)	7.0 (± 0.9)	6.9 (± 0.5)
TNF-α	6.0 (± 0.4)	4.8 (± 0.5) **	6.0 (± 0.4)	5.1 (± 0.5) **
IL10	8.0 (± 0.8)	5.6 (± 0.4) ** §§	8.4 (± 0.5)	6.4 (± 0.3) ** §§
CD14	4.3 (± 0.7)	3.1 (± 0.6) ** §§	4.2 (± 0.4)	4.3 (± 0.5) §§
TLR4	4.0 (± 0.8)	2.9 (± 0.6) ** §§	4.2 (± 0.5)	4.0 (± 0.4) §§

[A]Animals sorted by disease outcome a described in M&M. Die n = 12; Survive n = 8.
[B]Data from PBMC collected prior to BHV-1 infection.
[C]Data from PBMC just prior to *M. haemolytica* challenge.
[D]Data are represented as mean ΔCt (± SD). A smaller ΔCt equates to more abundant gene expression.
** = $P < 0.005$ vs day 0.
§§ = $P < 0.005$ vs other group's day 4.

expression in PBMC, however, provides evidence that IFN-γ production in the upper respiratory tract had a systemic effect and could therefore contribute to the increased expression of the TLR4 signaling complex.

Discussion

Epidemiological studies implicate stress as a factor contributing to viral-bacterial synergy and fatal secondary bacterial respiratory infections in cattle,[28,29] but the relative importance of stress in this disease complex has not been determined. Psychological stressors significantly alter immune responses to experimental viral respiratory infections, but these models have not been used to determine if stress significantly affects secondary bacterial infections of the respiratory tract. The present investigation used a combined viral and bacterial respiratory disease model to analyze the impact of combined psychological and nutritional stressors on BRD. A remarkable observation was that these stressors resulted in a doubling of fatal BRD when compared to calves experiencing transport alone (Figure 1). The rapid onset of death and limited lung pathology suggested that a systemic reaction, such as septic shock, rather than increased bacterial colonization of the lung was the cause of increased mortality in AW calves. This conclusion was supported by a variety of clinical and molecular analyses which revealed increased pro-inflammatory responses during viral infection and immediately prior to secondary bacterial challenge.

There is increasing evidence that respiratory viral infections can modulate the expression of receptors involved in responses to LPS derived from gram-negative bacteria. It was recently reported that blood monocyte expression of TLR4 was increased following human respiratory syncytial virus (RSV) infection of young children,[21] and infection with porcine reproductive-respiratory syndrome (PRRS) virus increased expression of both CD14 and LPS binding protein in the lung.[43] Our functional genomic analyses also revealed BHV-1 infection increased PBMC expression of both CD14 and TLR4 and increased expression of these receptors significantly correlated with fatal BRD (Table 2). Thus, modulation of TLR4 and associated adaptor molecule expression by a primary viral infection may be a general mechanism to enhance viral-bacterial synergy with secondary gram-negative bacterial respiratory infections. We also observed increased expression of TLR2, the receptor for peptidoglycans, following BHV-1 infection (data not shown). Thus, primary viral respiratory infections may enhance pro-inflammatory responses to both gram-negative and gram-positive bacterial infections by enhancing innate immune responses.

It is critical to understand mechanisms by which viral respiratory infections enhance TLR expression to effectively modulate stress responses and prevent fatal secondary bacterial infections. BHV-1 is a potent inducer of IFN-α and IFN-γ secretion in the upper respiratory tract,[7,36] and both cytokines have been implicated as mediators of LPS-sensitization following viral infection.[17,35] Earlier studies did not identify the mechanism by which IFNs induce LPS-sensitization, but our observations and other studies[41] support the conclusion that this sensitization occurs through increased TLR4 signaling complex expression. Thus, significant correlations between both IFN-γ secretion and mortality and the expression of TLR4 and CD14 and mortality may reveal a causal relationship between antiviral responses and enhanced viral-bacterial synergy. Prior analysis of blood leukocyte populations during BHV-1 infection revealed blood monocyte numbers remain relatively constant during infection.[7] Thus, increased expression of TLR4 and CD14 expression cannot be simply explained by a monocytosis following viral infection. A possible link between IFN secretion in nasal secretions and altered gene expression in PBMC was supported by the increased 2'5' OAS gene expression in these cells (Table 1). Furthermore, a significant correlation between 2'5' OAS and mortality (Table 2) provided independent confirmation that viral-induced IFN may contribute to fatal viral-bacterial synergy. A high correlation between IFN-α and IFN-γ levels in nasal secretions of BHV-1 infected calves makes it difficult to determine if one or both of these IFNs may contribute to increased TLR4 and CD14 expression.

If viral-induced IFN is a key factor contributing to increased mortality in AW calves, then a question arises as to how stress significantly enhances IFN-γ secretion following BHV-1 infection (Figure 3B). Gene expression analysis revealed IFN-γ expression did not change in PBMC of animals in both experimental groups (Table 1). This observation may be consistent with a previous report that BHV-1 infection did not activate NK cell activity in blood, but did result in the rapid recruitment of active NK cells to the site of infection.[13] Thus, enhanced recruitment and activation of NK cells in the respiratory tract of AW calves may be one mechanism by which stress could increase IFN-γ secretion. Specific psychological stressors, such as restraint, can inhibit leukocyte migration to the murine lung,[16,27,38] but social reorganization increased leukocyte migration into the lung and immunopathology.[39] Stressors associated with abrupt weaning may enhance fatal viral-bacterial synergy through an indirect effect on NK cell recruitment to the site of viral infection. This hypothesis would be consistent with the observation that similar levels of BHV-1 replication were observed for both experimental groups (Figure 3A) since BHV-1 is highly resistant to the antiviral effects of IFN.[3]

Pro-inflammatory responses induced by a primary BHV-1 infection have been implicated in the viral-bacte-

rial synergy of a fatal *M. haemolytica* infection.[9] Fever and elevated serum haptoglobin levels in the AW group (Figure 2) may be explained by either increased IFN production[8,20] or increased production of pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α.[22] Expression of pro-inflammatory cytokine genes in PBMC did not support the conclusion that these cytokines were important mediators of inflammation during primary viral infection. TNF-α expression was elevated in all animals, but there was no correlation with fatal viral-bacterial synergy (Table 2). One limitation of functional genomic analyses, as observed with IFN-γ, is that changes within blood leukocytes may not reflect host responses at the site of viral infection. Therefore, a more complete evaluation of the role pro-inflammatory cytokines play in viral-bacterial synergy will require an analysis of cytokine gene expression or protein levels at the site of viral infection. Significantly increased IL-10 expression in PBMC from animals with fatal viral-bacterial synergy (Table 2) may indicate that pro-inflammatory cytokines do not contribute to fatal BRD. IL-10 is a potent anti-inflammatory cytokine and can suppress both TLR4 signaling and pro-inflammatory cytokine production.[24,37] Thus, development of an acute systemic reaction in animals with increased IL-10 expression levels may indicate that fatal viral-bacterial synergy involves a complex dysregulation of the pro-inflammatory response.

Conclusions

The present investigation provides the first experimental evidence that a combination of psychological and nutritional stressors associated with abrupt weaning significantly enhanced fatal BRD. These stressors appeared to have a direct effect on the amplitude of antiviral responses, specifically the production of IFN at the site of viral infection. The surprising result is that these stressors enhanced, rather than inhibited, innate immune responses to BHV-1 infection. Future studies will need to determine if these stress-induced responses are unique to abrupt weaning and BHV-1 infection or occur with other viral respiratory infections and other types of stressors. Functional genomic analyses suggest antiviral responses were linked to an increased capacity to respond to gram-negative bacterial respiratory infections through increased expression of TLR4 and CD14. These analyses provide evidence for a novel mechanism by which stress may enhance the risk of fatal BRD. In addition, modulation of TLR expression during viral infection may be of relevance for both gram-negative and gram-positive bacterial infections, since TLR2 expression was also increased following BHV-1 infection. Further investigations will be required, however, to determine if other components of the TLR signaling pathway are also altered following viral infection. These molecular analyses should facilitate the identification of appropriate therapeutic targets for the prevention or treatment of stress-enhanced BRD infections.

Endnotes

[a]Model 099 HD, DeVilbiss, Somerset, PA
[b]MEM; GibcoBRL
[c]Euthanyl, Biomeda MTC, Cambridge Canada
[d]Micotil; Eli Lilly Canada Inc., Toronto, Canada
[e]Vacutainer® Preanalytical Solutions, NJ USA
[f]Invitrogen Canada, Inc., Burlington, ON, Canada [g]Qiagen Inc. Canada, Mississauga, ON, Canada
[h]Agilent Technologies Canada Inc., Mississauga, ON, Canada
[i]Invitrogen Canada Inc.
[j]SciEd Software
[k]GraphPad Software, Inc., San Diego, CA

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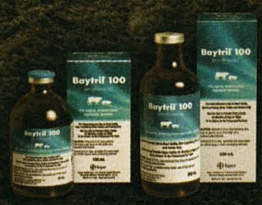

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