Effects of Lipopolysaccharide and *Mannheimia haemolytica* Leukotoxin on Bovine Lung Microvascular Endothelial Cells and Alveolar Epithelial Cells

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Bovine respiratory disease resulting from infection with *Mannheimia haemolytica* commonly results in extensive vascular leakage into the alveoli. *M. haemolytica* produces two substances, lipopolysaccharide (LPS) and leukotoxin (LKT), that are known to be important in inducing some of the pathological changes. In the present study, we examined bovine pulmonary epithelial (BPE) cell and bovine lung microvascular endothelial cell monolayer permeability, as measured by trans-well endothelial and epithelial cell electrical resistance (TEER), after incubation with LPS, LKT, or LPS-activated neutrophils. Endothelial cell monolayers exposed to LPS exhibited significant decreases in TEER that corresponded with increased levels of proinflammatory cytokines, apoptosis, and morphological changes. In contrast, BPE cells exposed to LPS increased the levels of production of inflammatory cytokines but displayed no changes in TEER, apoptosis, or visible morphological changes. Both cell types appeared to express relatively equal levels of the LPS ligand Toll-like receptor 4. However, TEER in BPE cell monolayers was decreased when the cells were incubated with LPS-activated neutrophils. Although the incubation of BPE cells with LKT decreased TEER, this was not reduced by the incubation of LKT with a neutralizing antibody and was reversed when LKT was preincubated with the LPS-neutralizing compound polymyxin B. Because BPE cells did not express the LKT receptor CD11a/CD18, we infer that contaminating LPS was responsible for the decreased TEER. In conclusion, LPS triggered changes in endothelial cells that would be consistent with vascular leakage, but neither LPS nor LKT caused similar changes in epithelial cells, unless neutrophils were also present.

Pneumonia caused by gram-negative bacteria in food animal species is an important disease, both economically and in terms of animal welfare. Organisms in the family *Pasteurellaceae* are frequently associated with pneumonia in several food animal species. Among the members of the family *Pasteurellaceae*, *Mannheimia haemolytica* is the organism that is the most commonly isolated from the lungs of cattle and sheep with severe respiratory disease (1, 9). A common element in all pneumonias caused by gram-negative bacteria, whether they occur in animals or humans, is the presence of lipopolysaccharide (LPS) in the lungs.

Acute pneumonia caused by *M. haemolytica* is characterized by infiltration of the airways with an inflammatory exudate that consists of neutrophils, fibrin, and blood (1, 9). The etiology of this acute vascular leakage in lung airways is controversial. *M. haemolytica* produces two major virulence factors, LPS and leukotoxin (LKT). It has previously been shown that LPS is directly cytotoxic to bovine endothelial cells (35). Apoptosis of the endothelial cells lining the lung vasculature may not be the only component responsible for the vascular leakage associated with *M. haemolytica* pneumonia. The emigration and activation of neutrophils in the lung may also be significant contributors to vascular leakage. In one study, the depletion of neutrophils in calves prior to inoculation with *M. haemolytica* reduced the amount of lung parenchymal damage compared to that in control animals (36). In addition, neutralization of the chemokine interleukin-8 (IL-8) in calves prior to inoculation with *M. haemolytica* significantly reduced the protein level in bronchoalveolar lavage fluid samples recovered from animals within the first few hours after infection (29).

For blood products to enter the alveoli and other airways, they must transverse the epithelial cells lining these structures. The effects of either LPS or LKT on bovine epithelial cells in the lung have not been well described. Histologic evaluation of calves 6 h after inoculation with *M. haemolytica* revealed effacement and a possible increase in the number of type II pneumocytes (epithelial cells) in the alveoli. In the same study, calves that were neutrophil depleted prior to infection had a lesser degree of degenerative changes in the epithelial cells lining the lung (9). Whether LPS has a direct effect on lung epithelial cells (i.e., activation or apoptosis) is questionable. The answer may depend in part on the types and the locations of the epithelial cells in the lungs. For example, in human lungs the epithelial cells lining airways are relatively nonresponsive to LPS, whereas type II pneumocytes lining the alveoli are
activated by LPS (3, 20). To the best of our knowledge, the effects of LPS and LKT on bovine lung epithelial cells have not been studied previously.

The present study examined the effects of both LPS and LKT on the permeability, morphology, and levels of apoptosis in bovine lung microvascular endothelial cells and alveolar epithelial cells. Our results suggest that endothelial cells, but not epithelial cells, are sensitive to the apoptotic effects of LPS. The levels of Toll-like receptor 4 (TLR-4) expression by both cell types were similar, suggesting different effects on LPS-stimulated signaling pathway or the lack of accessory molecules needed for LPS stimulation by the epithelial cells. In contrast, nether cell type underwent apoptosis in response to LKT, nor did they express the CD11a/CD18 receptor for LKT.

MATERIALS AND METHODS

Endothelial cells. Bovine lung microvascular endothelial (BPMEC) cells were commercially acquired from CS-C Cell Systems (Kirkland, WA). The cells were grown on T-25 tissue culture flasks (Falcon; BD Biosciences, Franklin Lakes, NJ) or in Dulbecco's modification of Eagle's medium (Cellgro; Mediatech Inc., Herndon, VA) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 IU penicillin–100 μg/ml streptomycin (Cellgro; Mediatech), 2 mM glutamine (Mediatech), and 1 μg/ml insulin (Sigma, St. Louis, MO). An inverted microscope with phase-contrast objectives (Diaspot, Nikon, Japan) and a digital camera were used to photograph the morphology of representative cells.

Isolation of lung epithelial cells. Bovine pulmonary epithelial (BPE) cells were isolated from a yearling Holstein-cross steer at the time of slaughter. The lungs were aseptically removed from the thoracic cavity of the animal, and the right accessory lung lobe was located and dissected from the remainder of the lung tissue. Several strips of lung (approximately 5 mm by 30 mm) were incised from the distal edge of the lung lobe and immediately placed into a 50-ml conical tube containing Dulbecco's phosphate-buffered salt solution (Fisher, Fair Lawn, NJ). The strips were further cut into smaller pieces, and the pieces were washed three times with medium. The tissue was digested for 20 min in 20 ml medium with 1% protease (Sigma). At 37°C the digested samples were filtered through filters with pore sizes of 2 μm, 150 μm, and 20 μm. The final filtrate was centrifuged at 100 × g for 10 min. The pellet was resuspended in 100 ml medium, which was then layered on 1,035-g/ml and 1,09-g/ml Percoll gradients (Amer sham Bioscience, Piscataway, NJ) and centrifuged for 20 min at 100 × g. The cells at the interface of the two Percoll gradients were aspirated, transferred to new tubes, and washed twice.

One milliliter of cells was then added to three wells of a six-well tissue culture plate (Falcon; BD Biosciences) and incubated for 1 h at 37°C. The nonadherent cells were gently removed, and the adherent cell monolayer was washed and trypsin-EDTA (Cambrex, East Rutherford, NJ) was used to determine the level of LPS contamination of the isolates from which LKT was isolated. The isolated cells were treated with a commercial kit (LKT, Ltd.) to remove LPS from LKT and mutant LKT were isolated from the conditioned media (Falcon). The cells were then treated with medium, 200 mM staurosporine, or one or 5 μg/ml LPS for either 12 or 24 h. The cells were then detached from the plate by trypsin-EDTA, washed twice with cold PBS, and resuspended in 100 μl of the buffer provided in the kit. A total of 10 μl annexin V-FITC and PI (5 μl each) was added to the cells, and the mixture was incubated for 15 min at 20 to 25°C. For each treatment, the fluorescence of 10,000 cells was measured by using a FACScanCaliber (BD Biosciences) flow cytometer. A four-quadrant region was created by using the fluorescence results for the individual annexin V, or PI-stained control cells. This region setup was used to analyze the control cells and the cells receiving various treatments. The percentage of cells in the upper right quadrant (double positive for annexin V and PI staining) and the lower right quadrant (single positive for annexin V staining) were determined. The experiments were repeated three times.

TLR-4 and CD11a/CD18 expression. An end-point PCR was used to determine the levels of mRNA for both TLR-4 and CD11a/CD18 in BPE, BPMEC, and BL-3 cells and neutrophils. The extraction of mRNA from the cells was performed by using a Qiagen RNeasy kit (valencia, CA) by the protocol supplied by the manufacturer. The concentration and purity of the mRNA were determined by measuring the absorbance of the samples at 240 and 260 nm with pH adjusted to 7.5. The cells were washed three times with sterile phosphate-buffered saline (PBS) and resuspended in Dulbecco's modification of Eagle's medium. The total cell counts and viability were determined by use of a hemocytometer and trypan blue exclusion. A differential cell count was used to determine cell purity. Only cell suspensions with greater than 95% neutrophils were used in the experiments.

Preparation of LKT. LKT and mutant LKT were isolated from M. haemolytica strain A1 and M. haemolytica strain SH1562 (a gift from S. V. Highlander, Baylor College of Medicine, TX), respectively, as described previously (4). Strain SH1562 carries a mutation in the b3K gene that inactivates LKT, rendering it noncytolytic. Brieﬂy, M. haemolytica A1 was inoculated into 200 ml of brain heart infusion broth with 0.5% yeast extract, which was incubated for 2 h at 37°C with agitation. The bacterial culture was centrifuged to collect cells at 4,100 × g for 10 min, and the supernatant was removed. The pellet was suspended in 200 μl H2O and was then lyophilized with the Speed-Vac apparatus. The LKT was reconstituted in PBS prior to use in the present study. The biological activity of the M. haemolytica LKT was determined by comparing it to the activity of the commercial E. coli LPS by the Limulus amebocyte lysate assay (Cambre, Walkersville, MD). Polymyxin B was purchased from MP Biomedicals (SOLON, OH). Staurosporine was obtained from Biomol Research (Plymouth Meeting, PA).

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a spectrophotometer (SmartSpec 300; Bio-Rad). mRNA (1.5 × 10⁶ H9262 cells) of 1:5,000 and a secondary goat anti-mouse HRP-labeled antibody (Santa Cruz) was used to locate specific bovine complete coding sequences for the genes of interest. Primers with a melting point of 60°C and an amplicon length of between 400 and 500 bp, which were obtained by use of the bovine gene sequences, were designed by using the software program Primer Express (version 3; Applied Biosystems, Foster City, CA). The primers were manufactured at Integrated DNA Technologies (Coraville, IA). PCR was performed on a thermocycler (PTC-200; MJ Research, Waltham, MA). After amplification, the samples were electrophoresed on a 1.5% agarose gel (PS00X2 power supply; Hoefer Scientific, San Francisco, CA). The gels were stained in ethidium bromide and visualized with a transilluminator (Fotodyne, Hartford, WI), and the images were recorded with a computer-documented digital camera.

**Western blotting.** Western blotting was performed with lysates of BPE, BPMEC, and BL-3 cells to identify TLR-4 protein expression. The cells were lysed in the lysis buffer by using the M-per reagent (Pierce) with 1 µg/ml protease inhibitor (Halt protease inhibitor; Pierce). The protein concentrations for the isolates and bovine serum albumin standards were determined by a bicinchoninic acid assay (Pierce), and the color development was read with a plate reader (DTX 880; Beckman Coulter, Fullerton, CA). Forty micrograms of protein from the samples and 4 µl loading buffer were first boiled for 5 min and then loaded onto a 7.5% Tris-HCl gel (Pierce). The samples were then electrophoresed on a 15 h at 100 V (Bio-Rad). Immediately following this, the protein was transferred to a nitrocellulose membrane (Transblot; Pierce). The membrane was blocked overnight. The TLR-4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the blocking solution at a concentration of 1:800 for 1 h at 22 to 25°C, and then the membrane was washed three times in Tris-buffered saline (TBST; 8.8 g/ml NaCl [Sigma], 0.2 g/ml KCl [Sigma], 3 g/ml Tris base [Sigma], and 0.05% Tween 20 [Sigma]; the pH was adjusted to 7.4), and then horseradish peroxidase (HRP)-labeled donkey anti-goat antibody (Santa Cruz) was added at a concentration of 1:3,000 for 1 h at 20°C before washing the membrane. The membrane was washed three times with TBST, and then reblocked overnight. The membrane was then probed for actin expression by using a mouse anti-actin primary MAb (Sigma) at a concentration of 1:5,000 and a secondary goat anti-mouse HRP-labeled antibody (Santa Cruz) at a concentration of 1:3,000. The remainder of the procedure was similar to that used for the probed of TLR-4.

**Flow cytometry.** Flow cytometry was used to identify CD11a/CD18 expression by BPE, BPMEC, and BL-3 cells. The cells were fixed in 4% paraformaldehyde (Sigma) for 20 min and washed three times in PBS with 1% bovine serum albumin. The cells were resuspended in PBS with bovine serum albumin and were incubated with 1:800 mouse anti-ovine CD11a antibody (VMRD, Pullman, WA) for 30 min at 4°C, washed three times in PBS with bovine serum albumin, and then incubated with FITC-labeled goat anti-mouse antibody (Jackson Immunoresearch, West Grove, PA) for 30 min at 20 to 25°C. The cells were then washed an additional three times with PBS and then analyzed with a flow cytometer (BD Biosciences). The histograms of the cell fluorescence were then displayed and overlaid on each other to combine the three graphs into one chart.

**Inflammatory mediator production.** A real-time reverse transcription-PCR (RT-PCR) was used to determine the changes in the levels of expression of the inflammatory mediators IL-1α, IL-1β, IL-8, and tumor necrosis factor alpha (TNF-α) in BPE and BPMEC cells after exposure to medium or 5 µg/ml LPS for 1 or 6 h. The cells were grown on six-well tissue culture plates, and the contents of two wells were combined for each treatment. After the incubation period, the cells were detached from the plate by using trypsin-EDTA and washed once in PBS, and the cell pellet was recovered. mRNA recovery and quantification were performed in a manner similar to that used for the end-point PCR, as described above. In addition, primer development was performed in a manner similar to that described above for the end-point PCR. The parameters for the development of the primers included an amplicon length of approximately 100 bp, a melting point of 60°C, a 30 to 80% GC content, and a primer length of between 9 and 40 bp. The primers were manufactured by Integrated DNA Technologies. Ninety-six-well optical reaction plates (MicroAmp; Applied Biosystems) were loaded with 150 µM primer sets, a 1:250 dilution of cDNA from the RT-PCR, and a 1:2 dilution of a SYBR green-containing master mixture (ABGene, Rochester, NY). The template amplification and measurement of the amplicon concentration were performed in a real-time PCR thermocycler (model 7300; Applied Biosystems). The software program Sequence Detection (version 1.3; Applied Biosystems) was used to analyze the data by using relative quantification. Changes in mRNA levels between the sample obtained at time zero and the LPS-treated samples were calculated as the levels of increase. Samples from the real-time reactions were also electrophoresed on a 1.5% agarose gel to determine the specificity of the reactions (single bands for each sample). Four separate experiments were performed, and the data are presented as the mean increases ± standard errors of the means (SEM). The data were analyzed with a two-tailed t test.

**Transwell endothelial and epithelial cell electrical resistance (TEER).** BPE and BPMEC cells were grown to confluence on 8-µm-pore-size Transwell inserts (Becton Dickinson, Franklin Lakes, NJ). The integrity of the monolayer on the inserts was confirmed by performing an initial determination of monolayer resistance. Inserts containing the monolayers were aseptically placed into a chamber (EndOhm-6; World Precision Instruments, Sarasota, FL) containing two electrodes. One electrode was in the lid that extended into the medium in the insert, while the other was in the base of the chamber, underneath the insert, which contained 1 ml of medium. An ohmmeter (EVOM; World Precision Instruments) was used to measure the electrical resistance between the two electrodes. To be used in an experiment, inserts containing endothelial cells had to have a minimum resistance of 75 Ω and inserts containing epithelial cells had to have a minimum resistance of 150 Ω. The inserts had medium to which combinations of E. coli or M. haemolytica LPS, LKT, mutant LKT, or neutrophils were added. The plates with the inserts were incubated at 37°C in a 5.0% CO₂ atmosphere; and the electrical resistance was measured at 0, 3, 6, 12, 24, and 48 h. Experiments with all treatment groups were run in triplicate, and the experiments were repeated three times. The results for replicate samples were averaged, and the percent change from the no-treatment sample at the same time point was calculated. In additional experiments, a neutralizing MAb of LKT was incubated with LKT at a 1:1.5 dilution for 0.5 h before it was added to the inserts. In a similar experiment, LKT was incubated with 0.5 µg/ml polymyxin B for 0.5 h before it was added to the inserts.

**Statistics.** Mean values ± SEMs were calculated for the replicates in the various experiments. Data were analyzed by analysis of variance with the StatView SE+ software program (Abacus Concepts, Berkeley, CA). The Tukey-
Kramer test was then used to compare the means that were significantly different. Statistical significance was set at a $P$ value of $<0.05$.

RESULTS

Morphological changes and apoptosis occurred in LPS-treated BPMEC cells but not BPE cells. BPE and BPMEC cells were incubated with medium or medium containing 1 $\mu$g/ml E. coli or M. haemolytica LPS for up to 24 h. Microscopic examination of the cells was performed at 0, 3, 6, 12, and 24 h. There were no apparent morphological changes in the BPE cells at any of the time points (Fig. 1). In contrast, BPMEC cells incubated with the LPS of either species exhibited obvious changes in morphology, especially at the 24-h time point. A moderate number of BPMEC cells detached from the plate, while the remaining attached cells were round and shrunken and a few displayed a spindle-shaped morphology. The detached cells and shrinkage of the remaining attached cells exposed large areas of the surface of the plate. Because a relatively pure form of E. coli LPS was commercially available, we decided to use it rather than our M. haemolytica-derived LPS in our studies. Our purpose in doing so was to minimize the possibility of introducing other contaminants into our sample. Side-by-side comparisons of the LPS from E. coli and that from M. haemolytica did not reveal any obvious differences in the BPMEC cell responses (data not shown).

Cells incubated for 12 or 24 h with LPS (1 or 5 $\mu$g/ml) were stained with annexin V-FITC or PI and were examined by flow cytometry to determine the numbers of cells undergoing apoptosis and necrosis. BPE cells exposed to LPS exhibited no change in the number of cells classified as being in either early apoptosis or late apoptosis/necrosis (Fig. 2). As a positive control, BPE cells exposed to staurosporine exhibited a significant increase in the number of cells classified as being in early apoptosis at 12 h and a significant increase in the number of cells classified as being in early apoptosis or late apoptosis/necrosis at 24 h. In contrast, BPMEC cells incubated with LPS displayed a significant number of cells that were classified as early apoptotic at 12 and 24 h. The numbers of BPMEC cells that were classified as late apoptotic/necrotic were also significantly elevated at 24 h.

TLR-4 expression by BPMEC and BPE cells. Because BPE and BPMEC cells responded differently to LPS, we next determined whether this difference was due to the differential expression of the LPS receptor TLR-4. TLR-4 mRNA levels were relatively equal between the two cell types, as measured by use of the end-point PCR (Fig. 3). The levels of TLR-4 mRNA in neutrophils were included as a positive control. Western blotting for TLR-4 confirmed that BPE and BPMEC cells appeared to have relatively equal levels of the TLR-4 protein.

Cytokine expression by BPMEC and BPE cells exposed to LPS. Since BPMEC and BPE cells appeared to express similar levels of TLR-4, we used a real-time RT-PCR to determine if BPMEC or BPE cells responded with similar levels of cytokine expression following activation by LPS. IL-1$\alpha$ mRNA levels...
were significantly elevated in both cell types after a 1- or 6-h exposure to LPS (Fig. 4). IL-1α and IL-1β mRNA levels were higher in the BPMEC cells than in the BPE cells at 6 h. IL-8 mRNA levels were also significantly increased in both cell types at both time points, although the IL-8 mRNA levels were nearly 10-fold higher in the BPE cells than in the BPMEC cells at either time point. In contrast, the TNF-α mRNA levels differed between the two cell types. BPE cells had a moderate increase in TNF-α mRNA levels at both time points, whereas BPMEC cells had virtually no increase at 1 h, and at 6 h the levels were increased only 0.5-fold over the level at the baseline.

**BPMEC and BPE cell monolayer TEER after exposure to LPS.** We next evaluated whether exposure to LPS altered the permeability of BPE and BPMEC cell monolayers using TEER as a measure of monolayer permeability. Incubation of BPE cells with 5 μg/ml LPS for up to 24 h did not result in any significant changes in TEER compared to that for the control monolayers (Fig. 5). In contrast, BPMEC cells incubated with LPS exhibited a slight decrease in TEER at 12 h, and this decrease became significant at 24 h. Staurosporine, which induces apoptosis in both BPMEC and BPE cells, caused an approximately 30% decrease in TEER in both cell types at 24 h. Incubation of neutrophils with either medium or LPS before coincubation with BPE cells resulted in a significant decrease (approximately 20%) in TEER. In contrast, BPMEC cells coincubated with LPS-activated neutrophils exhibited a significant decrease in TEER, whereas coincubation with neutrophils alone had no effect.

The *M. haemolytica* LKT is a critical virulence factor that impairs host leukocytes (40, 43). There is little or no information about what effect LKT might have on lung epithelial or endothelial cells. Incubation of BPMEC cells with LKT caused a small decrease in the cell monolayer TEER at 24 h that became significant (50% reduction) at later time points (Fig. 6). The effect of LKT was dose dependent (data not shown). BPE cells exposed to LKT exhibited a small decrease in TEER at 48 and 72 h, but this decrease was not statistically significant. We addressed the possibility that LPS contamination of the LKT might cause the decreased TEER in the BPMEC cells. When LKT was incubated with the neutralizing antibody MM601 prior to incubation with the BPMEC cells, the decrease in TEER was still observed (Fig. 7). However, incubation of LKT with the LPS-neutralizing agent polymyxin B prior to addition to the culture inserts prevented the decrease in TEER. As a further con-

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**FIG. 2.** Increased early apoptosis and necrosis/late apoptosis of BPMEC cells but not BPE cells incubated with LPS for 12 and 24 h. BPE and BPMEC cells were incubated with medium, staurosporine, or 1 or 5 μg/ml LPS for up to 24 h. The cells were recovered, washed, and stained with both annexin V and PI. Cells expressing fluorescence with either stain were counted with a flow cytometer, and the percentage of cells that were positive for staining was calculated for the BPMEC cells at 12 (A) or 24 h (B) or for the BPE cells at 12 (C) or 24 h (D). The values shown represent the means ± SEMs of three separate experiments. *, a P value of ≤0.05 compared to the results for the medium-treated controls.
control, we used an inactive LKT produced by an \textit{lktC} mutant of \textit{M. haemolytica} that was prepared in a similar manner as the wild-type LKT. Incubation of the BPMEC cell monolayer with this mutant LKT also resulted in a decrease in TEER, leading us to infer that contaminating LPS, rather than LKT itself, was responsible for the decrease in the BPMEC cell TEER.

\textbf{CD11a/CD18 expression by BPE and BPMEC cells.} To clarify further any possible role for LKT, we examined the expression of its receptor, LFA-1 (CD11a/CD18), by BPE and BPMEC cells (19). We first examined the CD11a and CD18 mRNA levels in BPMEC and BPE cells using an endpoint PCR. Neither cell type had measurable CD11a or CD18 levels (Fig. 8). As a positive control, we confirmed that the bovine lymphoblastic cell line BL-3, which is responsive to LKT, had high levels of mRNA for both CD11a and CD18 (4). Similar results were obtained by flow cytometry. Neither BPE cells nor BPMEC cells expressed appreciable levels of the CD11a protein on their surfaces. In contrast, BL-3 cells expressed a 10-fold higher level of CD11a compared to the level expressed by either the BPMEC cells or the BPE cells.

\textbf{DISCUSSION}

The events that lead to the extensive lung pathology associated with \textit{M. haemolytica} pneumonia in calves are not well defined. In the present study, we examined the effects of the primary virulence factors of \textit{M. haemolytica}, LPS and LKT, on two major cell types (epithelial and endothelial cells) in the lung. As previously demonstrated with endothelial cells from other vessels (21, 27, 37), microvascular endothelial cells were sensitive to LPS, undergoing apoptosis within 12 h of exposure. We have previously demonstrated a similar effect in pulmonary artery endothelial cells incubated with the related cell wall component lipooligosaccharide obtained from \textit{Haemophilus somnus} (38). Several other groups have also demonstrated apoptosis in bovine endothelial cells after exposure to LPS in vitro (7, 14). Apoptosis was not the only reaction that occurred in the BPMEC cells exposed to LPS. They also displayed increased levels of mRNA for several inflammatory cytokines. In particular, there was a substantial elevation in IL-8, which has previously been demonstrated to be a potent chemoattrac-
tant for bovine neutrophils (10, 25). Similar increases in IL-8 production have been demonstrated in human umbilical vein endothelial cells and coronary artery endothelial cells exposed to LPS in vitro (26, 46).

It is likely that the BPMEC cell response to LPS is dependent on the expression of TRL-4 by the endothelial cells. The pathway for TLR-4 signaling that has been the best described involves the adapter protein MyD88, which results in translocation and activation of NF-κB in the cell nucleus (8, 16). NF-κB translocation, in turn, is associated with the expression of several proinflammatory cytokines (TNF-α, IL-1β, IL-8) and adhesion molecules (VCAM-1, ICAM-1, E-selectin) (11, 34, 41, 44). TLR-4 activation of endothelial cells also can initiate apoptosis within these cells, presumably through signaling molecules such as MyD88, IRAK, MAL, and TRAF-6 (5, 6, 17). In the present study, BPMEC cells exposed to LPS exhibited both morphological and chemical signs consistent with apoptosis.

Whether the effects of LPS on endothelial cells result in blood vessel leakage is not yet well resolved. One study that used human umbilical vein endothelial cells exposed to LPS-conditioned plasma demonstrated a significant level of albumin leakage across the endothelial monolayer within 2 h of exposure, but not apoptosis (33). In the present study, significant changes in monolayer integrity, as measured by TEER, did not occur until 24 h of LPS exposure. This response corresponded in part with the increased levels of apoptosis among the BPMEC cells. Several factors could explain the temporal variance in the responses noted in the two studies. First and most obviously, the cells were obtained from different species and anatomical sites. Another likely explanation is that the LPS-conditioned plasma contained multiple inflammatory cytokines, produced by blood leukocytes, that triggered the rapid changes in the human umbilical vein endothelial cells. Consistent with this hypothesis, those authors prevented the change in permeability by the addition of neutralizing anti-TNF-α and IL-1β antibodies (33). Furthermore, the previous study used a different parameter to measure permeability (albumin leakage), while we assessed TEER.

We were unaware of previous investigations of the effects of LKT on BPE or BPMEC cells. Exposure of the BPMEC cells to M. haemolytica LKT induced a significant change in TEER that was first measurable at 24 h. Further investigation revealed that this response appeared to result from LPS contam-
ination of the LKT preparation. Preincubation of the LKT with an anti-LKT-neutralizing MAb did not prevent the decrease in TEER. Preincubation of the LKT with the lipid A-neutralizing compound polymyxin B prevented drops in TEER. To further clarify the apparent nonresponsiveness of the BPMEC cells to LKT, both end-point PCR and flow cytometry were performed with mRNA and protein samples from the cells. With neither technique did we find evidence that BPMEC cells express the LKT binding receptor CD18/CD11a (19). In contrast, BL-3 cells, which are sensitive to LKT, were used as a control for CD18/CD11a expression by both end-point PCR and flow cytometry (4). Both techniques demonstrated the ample expression of CD18/CD11a by BL-3 cells.

Neutrophils are known to make contributions to inflammation in the lungs of cattle with M. haemolytica pneumonia (2, 36). The involvement of neutrophils in microvascular damage and lung leakage during the early stages of M. haemolytica pneumonia in calves is controversial. We examined whether neutrophil activation results in TEER changes in endothelial cell monolayers that would be consistent with vascular leakage. Neutrophils did not appear to contribute to endothelial damage, as coincubation of BPMEC cells with LPS and neutrophils did not affect the drop in TEER observed after the LPS exposure. Several studies have shown a reduction in lung pathology in calves in which neutrophils were depleted prior to inoculation with M. haemolytica (36, 42). In contrast, a different study demonstrated no reduction in lung pathology when neutrophil depletion occurred prior to experimental M. haemolytica infection (9).

The BPE cells used in our study were relatively impervious to the apoptotic effects of LPS, as demonstrated by the lack of any visible morphological changes or changes in annexin V or PI staining following a 24-h incubation with LPS. Other studies have reported epithelial cell apoptosis following the LPS exposure of a cultured cell line or upper airway-derived epithelial cells (32, 39). However, both of those studies used a 100-fold larger amount of LPS (100 mg/ml) than we used in our study. The apparent “disconnect” between BPE cell activation and a
lack of apoptosis after exposure to LPS is currently under investigation. There are several possible explanations. Several studies have demonstrated that tracheobronchial epithelial cells express TLR-4, but much of this may be intracellular (15, 31). A different study demonstrated a deficiency in the TLR-4-associated molecule MD-2 in primary cultures of human airway epithelial cells. The overexpression of MD-2 in these cells conferred a hyperresponsiveness to LPS (20). Another study demonstrated a reduced level of TLR-4 expression by bronchial epithelial cells in LPS-treated rats compared to the levels of expression by lung endothelial cells, macrophages, and neutrophils. In addition, it was noted that most of the TLR-4 was located within the epithelial cells and was not on the cell surface (18). However, the intracytoplasmic localization of TLR-4 might not prevent LPS–TLR-4 interactions, as one study demonstrated potent LPS signaling via intracellular TLR-4 (12). LPS has been demonstrated to colocalize with TLR-4 in both the cytoplasm and the nucleus of lung cells recovered from rats intratracheally inoculated with LPS (18). In the present study, BPE cells expressed both mRNA and protein for TLR-4, although we did not determine whether it was in the cytoplasm or on the cell surface. The TLR-4 did appear to be functional, as the epithelial cells responded to LPS by increased cytokine production. These findings suggest that lung epithelial cells are not directly damaged by LPS but contribute to the inflammatory process by producing cytokines.

Similar to what was seen following LPS exposure, incubation of BPE cells with LKT resulted in only minor changes in permeability and cell morphology. When we examined the expression of the LKT receptor CD18/CD11a on BPE cells by end-point PCR and flow cytometry, it appeared that BPE cells, like BPMEC cells, express little or no CD18/CD11a. To the best of our knowledge, this is the first reported investigation of the potential effects of LKT on lung epithelial cells.

Because neither LPS nor LKT induced TEER changes in BPE cells that would be expected to result in the leakage of blood products into the alveoli, we also investigated the possible effects of neutrophil activation on this parameter. The BPE cell monolayer TEER was significantly reduced when the cells were coincubated with LPS and neutrophils. This observation is in agreement with previous reports of direct damage to lung epithelial cells by activated neutrophils (22, 23). This damage is likely due to the secretion of proteases and reactive oxygen species by the neutrophils, as studies that used neutrophil elastase or reactive oxygen species inhibitors prior to the induction of an insult in the lung showed reduced overall lung injury (13, 24, 30). In the present study, the coincubation of neutrophils with BPE cells without LPS activation also induced a decrease in the BPE cell TEER. Therefore, it would appear that neutrophils are involved in damage to lung epithelial cells that would likely result in the leakage of blood products into the alveoli. However, it would appear that neutrophils alone, with or without exposure to LPS, are sufficient in causing this response.

The results of this study indicate that LPS has a direct apoptotic effect on bovine lung microvascular endothelial cells that results in a decreased electrical resistance across monolayers of these cells. These changes would be consistent with the leakage of vascular products into the interstitium of the lung during pneumonia. In contrast, LPS exposure did not cause changes in apoptosis or electrical resistance in BPE cells. The presence of neutrophils, however, induced a decrease in the BPE cell monolayer TEER that would likely allow the leakage of blood products into the airspaces of the lung. Future studies will attempt to identify the mechanisms responsible for this differential response to LPS. Both cell types responded similarly to LPS stimulation by increased cytokine production. They also expressed similar quantities of mRNA and protein for the LPS receptor TLR-4. The LKT of M. haemolytica did not directly damage endothelial or epithelial cells in our study, probably in part because of their lack of expression of CD11a/CD18. Therefore, it is likely that a combination of LPS and neutrophils during M. haemolytica pneumonia results in the extensive leakage of vascular products into the airspaces so commonly associated with this disease.

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