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Cytokine expression by pulmonary leukocytes from calves challenged with wild-type and leukotoxin-deficient *Mannheimia haemolytica*

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ABSTRACT

The objective of this study was to assess the role of leukotoxin (LKT) in modulating the pulmonary cytokine response of calves challenged with *Mannheimia haemolytica*. Thirty-six calves, seronegative to LKT and *M. haemolytica* whole cell antigen were divided into three groups (I, II and III). Calves in groups I and II were challenged by the intra-bronchial route with 25 mL of phosphate buffered saline (PBS) containing 0.44×10^9 cfu/mL of LKT deficient (*lkt*$^-$) and 25 mL of PBS containing 0.31×10^9 cfu/mL of wild-type (wt) *M. haemolytica* serotype 1, respectively. Group III calves were challenged intra-bronchially with 25 mL of sterile PBS. Leukocytes were collected from broncho-alveolar lavage fluid (BALF) 4 days before and at 1, 3, and 6 days post-inoculation (p.i.). Expression of the following cytokines in the recovered leukocytes was measured using real-time PCR: interleukin (IL)-1β, -8, -10, -12 (p40) and TNF-α. The amount of TNF-α produced was also quantified by ELISA. Although a statistically significant difference in the expression of these cytokines was not observed between groups challenged with the wt and *lkt*$^-$ strains, the wt infected group (II) did exhibit higher mean clinical scores. Overall, there was considerable variation in the composition of the BALF between the groups and by day 7 p.i., both *lkt*$^-$- and wt-challenged calves had seroconverted to *M. haemolytica*.

Introduction

Mannheimia haemolytica is the major cause of the fibrinous and necrotizing pleuropneumonia of cattle termed bovine pneumonic pasteurellosis (BPP) or 'shipping fever'. Several virulence factors of *M. haemolytica* promote host-pathogen interactions by enabling bacterial colonization of the lungs and contribute to the development of pneumonia (Highlander, 2001). Leukotoxin (LKT) and lipopolysaccharide (LPS) are key factors in this context (Confer et al., 1990).

Most of the knowledge of the role of LKT and LPS in the pathogenesis of BPP has come from in vivo studies using purified LKT alone or in combination with LPS (Stevens and Czuprynski, 1995; Marcatili et al., 2002). Since the effects of LKT and LPS are synergistic, their roles are better appreciated when used in combination (Lafleur et al., 2001). In vivo studies using whole bacteria are limited, and, to date, only two studies using LKT deletion mutant organisms (*lkt*$^-$) have been conducted (Tatum et al., 1998; Highlander et al., 2000). These studies found reduced clinical and lung lesion scores in animals infected with *lkt*$^-$ mutants compared to those challenged with wild-type (wt) strains of *M. haemolytica*. Lower lesion scores were characterized by reduced necrosis and lower numbers of degenerate neutrophils (Tatum et al., 1998; Highlander et al., 2000). However, Highlander et al. (2000) could only demonstrate a partial reduction in the virulence of the *lkt*$^-$ mutant. The molecular basis of this attenuation remains to be elucidated.

Given the key role of inflammatory cytokines in the pathogenesis of BPP (Lafleur et al., 2001; Malazdrewich et al., 2001), a profile of the cytokines expressed in response to infection would likely enhance our understanding of the important events at play. In this study we chose to quantify tumor necrosis factor (TNF)-α and interleukin (IL)-1β and -8 as these are pleiotropic, early response pro-inflammatory mediators that are produced by a variety of cells (Malazdrewich et al., 2001). IL-10 and -12 were selected because of their role in regulating the adaptive immune response.

Our objective was to investigate the role of LKT in modulating cytokine gene and protein expression in bovine leukocytes obtained from the lungs of calves experimentally infected with isogenic LKT-deficient (*lkt*$^-$) and with wt strains of *M. haemolytica*

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serotype 1, respectively. The wt strain was isolated from a pneumonic bovine lung and was found to be more virulent than existing strains cultured from cattle in the Oklahoma region of the USA (unpublished data). The *lkt*⁻ strain was prepared by allelic replacement of the entire *lktA* and part of the *lktC* genes with the beta-lactamase enzyme gene (Murphy et al., 1995). A 'mixed regression' model was used in the hypothesis testing.

Materials and methods

Preparation of inoculum

Both the wt and *lkt*⁻ strains were cultured in a similar fashion and had almost identical growth curves. Briefly, bacteria were grown overnight on brain heart infusion (BHI) agar containing 5% sheep blood (Hardy Diagnostics), both without and with added ampicillin (10 μg/mL) for the wt and *lkt*⁻ cultures, respectively. Multiple colonies were subsequently transferred into 150 mL of BHI broth and incubated for 6 h at 37 °C at 70 oscillations/min (opm) in a shaking incubator. The broth culture was centrifuged twice at 8000 *g* for 15 min. The bacterial pellet was washed with 10 mL of phosphate buffered saline (PBS). After a second centrifugation, the pellet was re-suspended in RPMI 1640 media or PBS and the concentration quantified by spectrophotometry (Ultraspec 2000, Pharmacia). In our previous experiments, an optical density (OD) of 0.72–0.74 at 650 nm corresponded to a concentration of approximately 10^9 colony forming units (cfu)/mL (Confer et al., 2006). The exact concentration of the viable bacterial suspension was subsequently confirmed by standard colony count at various dilutions on BHI blood agar as 0.44×10^9 cfu/mL and 0.31×10^9 cfu/mL for the *lkt*⁻ and wt strains, respectively.

Leukotoxin production

Both wt and *lkt*⁻ strains were grown on BHI supplemented with 5% sheep blood agar. A single colony was suspended in BHI broth and incubated overnight at 37 °C in a shaking incubator at 160 opm. The culture was subsequently re-suspended in 1 L of RPMI 1640 (R7509 Sigma) and incubated at 37 °C at 160 opm. The culture was centrifuged at 3800 *g* for 20 min and the supernatant was filtered using 0.2 μm filters (Fisher 167-0020). The concentrate was then repeatedly centrifuged (Amicon ultra-15, Millipore) at 2000 *g* for 15 min. The culture supernatant containing semi-purified LKT was aliquoted and stored at −86 °C.

On Western blotting, a monoclonal antibody MM 605 (kindly provided by Dr. S. Srikumaran, Washington State University) recognized the 102 kDa LKT band and associated breakdown bands in the supernatant obtained from the culture of wt *M. haemolytica*, whereas a similar band was not detected in the culture supernatant obtained from *lkt*⁻ strain (Singh et al., 2011). The growth of the wt strain on 5% sheep blood agar was accompanied by a distinct zone of hemolysis surrounding the colonies. No such zone was associated with growth of the *lkt*⁻-strain (data not shown). Bovine alveolar macrophages (BAM) challenged with wt strain exhibited 43% more cytotoxicity compared to cells challenged with the *lkt*⁻ strain (Singh et al., 2011).

Experimental protocol

The study was conducted under the guidelines of Oklahoma State University Institutional Animal Care and Use Committee (protocol number VM50182).

Thirty-six, 140–200 kg, male and female, cross-breed calves were screened for antibodies against LKT and *M. haemolytica* whole cell (WC) antigens at 18 days prior to inoculation using an ELISA (Confer et al., 1985). Healthy calves with minimal antibody concentrations were selected, transported to the study site 10 days prior to the commencement of the experiment, and were allowed to acclimatize. Calves were randomly assigned to three groups (I, II and III) of 12 animals/group.

Group I and II calves were challenged intra-bronchially with *lkt*⁻ and wt *M. haemolytica* strains, respectively. Group III animals served as controls and were challenged with sterile PBS. Calves from the different groups were held in individual pens located so as to avoid the aerosol transfer of bacteria. On day 0, calves in groups I and II received 25 mL of suspension in PBS containing 0.44×10^9 cfu/mL of the *lkt*⁻ and 0.31×10^9 cfu/mL of the wt strains, respectively. These were delivered intra-bronchially using an endotracheal tube followed by a 25 mL PBS 'flush'. Bronchoalveolar lavage fluid (BALF) was obtained from each calf 4 days before infection and at 1, 3 and 6 days post-inoculation (p.i.) using 180 mL of sterile PBS via an endotracheal tube with the instilled fluid retrieved by suction. On average, less than 50% of the instilled fluid was collected in 50 mL tubes containing 1% penicillin–streptomycin and amphotericin B (Cambrex) and was transported to the laboratory on ice. The tubes were then centrifuged at 500 *g* for 7 min and 500 μL of supernatant was collected for ELISA. The cell pellet was gently washed in 10 mL of PBS and then centrifuged again in 15 mL of PBS at 500 *g* for 7 min. RNA was extracted from the cell pellet as previously described (Rottman et al., 1996).

From 1 day prior to inoculation to 6 days p.i., the calves were clinically evaluated daily and given a clinical 'score' based on a nonparametric scale (Tatum et al., 1998). Scores of between '0' and '4' were allocated based on the following criteria: rectal temperature >39 °C; evidence of depression; and dyspnea or respiratory rate ⩾60/min.

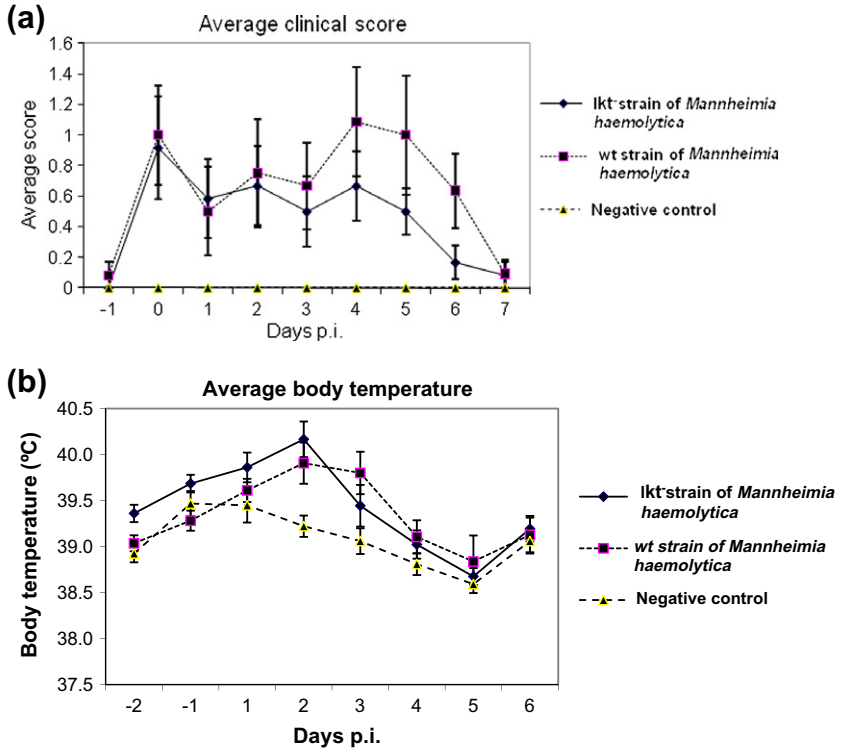

Fig. 1. Mean (±SE) clinical score (a) and body temperature (b) of calves challenged with wildtype (wt) and leukotoxin deficient (*lkt*⁻) strains of *Mannheimia haemolytica* and of uninfected negative control animals from prior to challenge until 7 days post-inoculation (p.i.).