Activation of Bovine Neutrophils by Partially Purified Pasteurella haemolytica Leukotoxin

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In this study we developed a new method for the partial purification of Pasteurella haemolytica leukotoxin by size-exclusion high-performance liquid chromatography. The partially purified leukotoxin had a molecular weight of 104,000, as estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and reacted on an immunoblot with an antileukotoxin monoclonal antibody. As expected, high concentrations of the leukotoxin were inhibitory or lethal to bovine neutrophils. Incubation of bovine neutrophils with diluted leukotoxin, however, resulted in significant neutrophil activation that was comparable in magnitude to that obtained with standard activating agents such as opsonized zymosan or zymosan-activated serum. Dilute leukotoxin (1:128 to 1:8,192 dilutions) stimulated an oxidative burst (luminol-dependent chemiluminescence) by bovine neutrophils that was comparable in magnitude to that obtained with opsonized zymosan. Preincubation with leukotoxin did not significantly prime the neutrophils for an enhanced oxidative burst when they were then exposed to opsonized zymosan as a second stimulus. Dilute leukotoxin (1:100 to 1:1,000 dilutions) also stimulated cytoskeletal alterations in bovine neutrophils, as measured by a significant shape change response. Preferential release of secondary granule constituents (lactoferrin) occurred when neutrophils were incubated with 1:100 to 1:500 dilutions of leukotoxin. Significant release of primary granules, as measured by β-glucosaminidase activity, was not observed except at low dilutions (1:20) of leukotoxin that resulted in significant release of cytosolic constituents (i.e., lactate dehydrogenase activity). The neutrophil-activating activity of the leukotoxin was heat labile, unaffected by polymyxin B, and abrogated by a leukotoxin-neutralizing monoclonal antibody. These data indicate that P. haemolytica leukotoxin, like the closely related Escherichia coli hemolysin, is a potent neutrophil-activating agent. Leukotoxin-stimulated release of neutrophil oxygen intermediates and granule constituents may contribute to the intense inflammation that characterizes bovine pulmonary pasteurellosis.

Pasteurella haemolytica is the principal bacterial pathogen of the bovine respiratory disease complex. In conjunction with stress or active viral infection, P. haemolytica causes an acute fibrinous pleuropneumonia (e.g., shipping fever) (16). P. haemolytica possesses several components that may function as virulence mechanisms (16, 36, 46). Foremost among these is the leukotoxin that it secretes during the logarithmic growth phase (1, 3, 39, 40).

Several investigators have shown that P. haemolytica leukotoxin shares considerable molecular homology with exotoxins produced by other gram-negative bacteria, including Escherichia coli (9, 10, 22, 33, 34, 42, 43), Actinobacillus pleuropneumoniae (20, 32), and Actinobacillus actinomycetemcomitans (30). Previous studies of the effects of P. haemolytica leukotoxin on bovine leukocytes have focused chiefly on its lethal or inhibitory effects (1, 3, 11–15, 19, 29, 39, 44, 45). Studies of the closely related E. coli hemolysin showed that, at low concentrations, it was a potent activator of human neutrophils (4, 5, 7, 8, 23, 24, 31). Similar studies of the P. haemolytica leukotoxin have not been reported, at least in part, because of technical difficulties in leukotoxin purification.

In this report we describe a high-performance liquid chromatography (HPLC) procedure that allows us to obtain partially purified leukotoxin that retains its biological activity. We then investigated whether sublethal concentrations of partially purified leukotoxin would stimulate bovine neutrophil oxidative activity, degranulation, and shape change. Our results indicate that the P. haemolytica leukotoxin, like the E. coli hemolysin, is a potent neutrophil-modulating agent.

MATERIALS AND METHODS

Production of crude leukotoxin. To prepare crude leukotoxin, P. haemolytica A1 (originally provided by R. Corstvet, Baton Rouge, La.) was inoculated onto blood agar and incubated at 37°C for 18 h. Isolated colonies were then used to inoculate brain heart infusion broth, which was incubated at 37°C for 4 h to allow the bacteria to enter the logarithmic growth phase. The bacteria were then harvested by centrifugation (1,000 × g for 15 min), suspended in RPMI 1640 tissue culture medium, and incubated at 37°C for 2 h. In our original preparations the RPMI 1640 was supplemented with iron-saturated bovine lactoferrin as described by Gentry et al. (25). We subsequently noted, however, that lactoferrin was not necessary to obtain leukotoxic activity. Therefore, it was not added in later experiments. After the 2-h incubation, the bacteria were removed by centrifugation (6,000 × g for 15 min) and the supernatant was filtered (0.45-μm-pore-size filter). Leukotoxic activity of the crude culture filtrate was determined by the automated colorimetric assay of Greer and Shewen (28) with neutral red uptake by bovine neutrophils as the target cells as described previously (19). One unit of leukotoxic activity was defined as the reciprocal dilution of leukotoxin that caused a 50% decrease in the neutral red assay.

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Leukotoxin-neutralizing MAb. The development and characterization of the leukotoxin-neutralizing monoclonal antibody (MAb) MM-601 used in this study was described previously (26). This immunoglobulin G1 antibody was used in the form of ascitic fluid or in 0.5 ml of Tris-500 mM acetate (pH 7.0) at a flow rate of 0.5 ml/min. Fractions were collected each minute, diluted 1:4 in RPMI 1640 with 10% fetal bovine serum, and assessed for leukotoxin activity against bovine neutrophils as described in Materials and Methods. The results of a single representative leukotoxin preparation are illustrated.

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Preparation of bovine neutrophils. Bovine neutrophils were purified from the peripheral blood of healthy Holstein cattle by flash hypotonic lysis and centrifugation through a Percoll gradient as previously described (18). Purified neutrophils were suspended at a final concentration of 10⁵ per ml in HBSS containing 5% fetal bovine serum (HBFS).

LDCL. The oxidative response of bovine neutrophils was measured by luminol-dependent chemiluminescence (LDCL) as previously described (19). Briefly, luminometer tubes containing 10⁵ neutrophils and 6.4 μM luminol (Sigma Chemical Co., St. Louis, Mo.) in 0.2 ml of HBFS were placed in a PicoLite model 6500 luminometer (Packard Instrument Co., Downer's Grove, Ill.). To determine direct stimulation, the cells were incubated with various concentrations of leukotoxin in 0.1 ml of HBSS. Chemiluminescence was measured for 5 s every 5 min for 1 h and was recorded as counts per second. To determine whether the leukotoxin was capable of priming bovine neutrophils, these same neutrophils were incubated with leukotoxin for 1 h and then stimulated with 0.1 mg of opsonized zymosan (OPZ) in 0.1 ml of HBFS. Chemiluminescence was determined as described above. All samples were run in triplicate and randomly distributed in the luminometer. Data presented are the mean ± standard error of the mean peak counts per second for 10⁵ neutrophils.

Neutrophil shape change. Neutrophil shape change was assessed as described previously (33). Briefly, 5 × 10⁵ neutrophils were suspended in serum-free HBSS that contained graded dilutions of leukotoxin (total volume of 0.1 ml) for 1 h at 37°C. Neutrophils incubated in 10% zymosan-activated bovine serum or HBSS alone were included as positive and negative controls, respectively. At the end of the incubation period, the neutrophils were fixed by the addition of 0.25 ml of cold 2% glutaraldehyde in phosphate-buffered saline. Individual cells (200 per suspension) were evaluated by light microscopy (×400 magnification) and scored as negative if they remained spherical and positive if they were no longer uniformly spherical or had formed pseudopodia.

Primary and secondary granule release. Primary granule release was determined by measuring the amount of N-acetyl-β-d-galactosaminidase activity in cell supernatants after incubation of neutrophils with leukotoxin for 1 h at 39°C as described previously (38). Serial dilutions of supernatants were added (0.02 ml) to triplicate wells of a microelisa plates (Flow Laboratories, Inc., McLean, Va.), and 0.03 ml of substrate (8 mM p-nitrophenol-d-glucosaminide and 0.5% Triton X-100 in 0.2 M citrate buffer [pH 4.0]) was added to each well. Plates were incubated at 39°C for 4 h, and the reaction was stopped by the addition of 0.2 ml of glycine-carbonate buffer (133 mM glycine, 83 mM sodium carbonate, 67 mM sodium chloride [pH 10.7]). The A₄₀₅ was determined with a Dynatech model MR 580 microelisa plate reader and converted to nanomoles of p-nitrophenol generated per hour based on a standard curve of known concentrations of p-nitrophenol. Data are expressed...
as the percentage of N-acetyl-β-D-glucosaminidase activity released as compared with the total activity present in a sonicated lysate of neutrophils from the same donor prepared on the same day.

Secondary granule release was determined by measuring the amount of lactoferrin present in neutrophil supernatants with a competitive immunosorbent assay that is specific for bovine lactoferrin (37, 38). Polyclonal rabbit antiserum raised against bovine lactoferrin was generously provided by W. Hurley (Champaign, Ill.) (37). The amount of lactoferrin in neutrophil supernatants was determined by comparing the absorbance in sample wells with those of known quantities of purified bovine lactoferrin (U.S. Biochemical Corp., Cleveland, Ohio). Data are expressed as the percentage of lactoferrin released by stimulated neutrophils as compared with that present in a sonicated lysate of neutrophils prepared from the same donor on the same day.

Electron microscopy. Neutrophils were incubated for 1 h at 39°C in serum-free HBSS (2 × 10⁶ cells per ml) that contained a 1:100 dilution of purified leukotoxin, in HBSS with leukotoxin to which a 1:10,000 dilution of the leukotoxin-neutralizing MAb MM-601 had been added, or in HBSS alone. The neutrophils were pelleted by centrifugation (400 × g for 10 min) and fixed with modified Karnovsky fixative. The neutrophil pellets were stained with 1% OsO₄, backstained with 1% uranyl acetate, and embedded in epoxy. Thin sections were prepared and examined with a Philips 410 electron microscope. Photographs were taken of representative neutrophils under each set of experimental conditions.

Statistical analysis. Statistical analysis of data was performed with a one-way analysis of variance followed by paired t tests by using the BMDP statistical package. Statistical significance was set at P < 0.05 for all comparisons.

RESULTS

Purification of leukotoxin. Leukotoxin was partially purified from crude culture supernatants by size-exclusion HPLC. Leukotoxic activity eluted in a discrete peak at approximately 16 to 19 min after the column was loaded (Fig. 1). SDS-PAGE analysis of partially purified leukotoxin indicated a doublet at approximately 104 kDa (Fig. 2A). The identities of the bands at approximately 66 and 7 kDa are unknown. Immunoblot analysis of purified leukotoxin with the leukotoxin-neutralizing MAb MM-601 revealed a similar doublet at approximately 104 kDa (Fig. 2B) without any detectable lower-molecular-mass bands. Other investigators have previously reported multiple bands in purified leukotoxin preparations (10, 26). The partially purified leukotoxin had an approximately 10-fold increase in leukotoxic units per milligram of protein as compared with crude leukotoxin (77,136 ± 22,930 and 7,433 ± 1,447 U/mg, respectively).

P. haemolytica leukotoxin directly stimulates bovine neutrophil chemiluminescence. When bovine neutrophils were incubated with sublethal concentrations of leukotoxin (1:128 to 1:1,996 dilutions), they underwent a significant (P < 0.01) LDCL response (Fig. 3A). This response was rapid, typically reaching its peak 4 to 8 min after the addition of leukotoxin (data not shown). We next determined whether preincubation with leukotoxin primed neutrophils for an enhanced LDCL response when subsequently exposed to a second stimulus like OPZ. When the same neutrophils illustrated in Fig. 3A were exposed to OPZ after a 1 h of incubation with leukotoxin, their LDCL response to OPZ was elevated only slightly (at toxin dilutions of 1:2,048 to 1:32,768) as compared with that of neutrophils preincubated in medium alone (P > 0.05) (Fig. 3B). Preincubation with leukotoxin sometimes accelerated the LDCL response to OPZ (4 to 12 min to peak counts per second at a 1:512 dilution of toxin versus 16 to 28 min for OPZ controls); however, this accelerated response was not observed in all experiments (data not shown). Preincubation with lower dilutions of leukotoxin (1:32 and 1:128) inhibited the LDCL response of the neutrophils to OPZ, similar to previous reports with crude leukotoxin (11, 12, 19).

P. haemolytica leukotoxin stimulates shape change of bovine neutrophils. Cytoskeletal alteration of neutrophils in response to various stimuli, such as chemoattractants, can be evaluated by their shape change (35). Incubation of neutrophils with sublethal concentrations of leukotoxin resulted in a significant shape change response that decreased as the leukotoxin was further diluted (Fig. 4). As a positive control, incubation of neutrophil suspensions with zymosan-activated bovine sera, a source of C5a and other chemoattractants, resulted in a significant shape change of virtually all neutrophils (Fig. 4).

P. haemolytica leukotoxin stimulates release of bovine neutrophil secondary granules. Purified leukotoxin stimulated the degranulation of secondary granules from bovine neutrophils as determined by secretion of lactoferrin (Table 1). Although some release of β-glucosaminidase activity was also detected, it was observed only at high concentrations (1:20 dilution) of leukotoxin that also elicited the significant release of lactate dehydrogenase activity, thus suggesting that β-glucosaminidase activity resulted, at least in part, from neutrophil lysis rather than from the secretion of primary granules (data not shown). Electron microscopy

![FIG. 2. SDS-PAGE and immunoblot analysis of partially purified leukotoxin](http://iai.asm.org/Downloaded)
confirmed that leukotoxin-treated neutrophils were swollen and vacuolated and released their granule constituents into the extracellular milieu (Fig. 5B).

The biological activity of partially purified leukotoxin is not due to contaminating lipopolysaccharide. Several lines of evidence indicate that contaminating lipopolysaccharide was not responsible for the biological activities of partially purified leukotoxin. Heating of leukotoxin at 70°C for 1 h completely abrogated and preincubation of unheated leukotoxin with polymixin B (10 μg) had no effect on the ability of leukotoxin to stimulate neutrophil chemiluminescence (log10 peak of 2.74 and 4.49 counts per second, respectively, as compared with 4.54 counts per second for the leukotoxin control). The addition of the P. haemolytica leukotoxin-neutralizing MAb MM-601 completely blocked the ability of leukotoxin to stimulate bovine neutrophil chemiluminescence, shape change, and degranulation (Table 2). In addition, transmission electron microscopy revealed that the MAb abrogated the ability of leukotoxin to cause neutrophil swelling and degranulation (Fig. 5C), resulting in cells that resembled control neutrophils that were not exposed to leukotoxin (Fig. 5A).

The results of this study indicate that P. haemolytica leukotoxin, like E. coli hemolysin, is a potent activator of neutrophils. Incubation of bovine neutrophils with purified leukotoxin directly stimulated a rapid oxidative burst, as measured by LDCL. Preincubation with leukotoxin had only a modest effect (P > 0.05) on the oxidative response of bovine neutrophils to subsequent stimulation with OPZ (Fig. 2). Leukotoxin also caused substantial cytoskeletal alterations (Fig. 3) and preferential release of secondary granules (Table 1). These results are similar to those of Bhakdi and co-workers (4), who reported that E. coli hemolysin caused a preferential release of secondary granules from human neutrophils. Although these workers did not observe stimulation of an oxidative burst (4), other investigators have reported that E. coli hemolysin causes chemiluminescence and release of specific reactive oxygen intermediates from human neutrophils (7, 8, 31). Our findings are consistent with the significant molecular homology between the P. haemolytica leukotoxin and the E. coli hemolysin (9, 10, 22, 33, 34, 42, 43).

Previous studies of P. haemolytica leukotoxin have fo-

**TABLE 1.** *P. haemolytica* leukotoxin stimulates release of secondary granules (i.e., lactoferrin) by bovine neutrophils

<table>
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<tr>
<th>Prepn</th>
<th>% Release of total activity* (mean ± SEM)</th>
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<tr>
<td></td>
<td>Lactoferrin</td>
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<td>Leukotoxin dilution</td>
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<tr>
<td>1:100</td>
<td>52 ± 17a</td>
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<tr>
<td>1:500</td>
<td>17 ± 3</td>
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<tr>
<td>1:1,000</td>
<td>6 ± 2</td>
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<tr>
<td>Control</td>
<td>3 ± 1</td>
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<td>OPZ</td>
<td>42 ± 8b</td>
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* Percent enzyme activity in the culture supernatants as measured in comparison with the enzyme activity in neutrophil lysates (100% activity). Results are from five separate experiments.

b P < 0.001 compared with results with control neutrophils.
FIG. 5. Electron microscopy of leukotoxin-treated neutrophils. Neutrophils (2 × 10⁶) were incubated for 1 h at 37°C with HBSS alone (A), a 1:100 dilution of purified leukotoxin (B), or a 1:100 dilution of leukotoxin with a 1:10,000 dilution of the leukotoxin-neutralizing MAb MM-601 (C). In the presence of leukotoxin alone, neutrophils were swollen and vacuolated and appeared to be in the process of releasing their granules. An extruded granule that is undergoing dissolution is indicated with an arrow (B). Control neutrophils (A) and neutrophils incubated with leukotoxin and the antileukotoxin MAb (C) had a similar morphologic appearance. Magnification, ×11,500.
cused principally on its inhibitory and lethal effects for bovine neutrophils (1, 3, 11, 12, 14, 19, 39). Most of these studies used crude culture supernatants that were subjected to little or no purification. One group of investigators succeeded in isolating relatively pure leukotoxin by anion-exchange chromatography and immunoprecipitation (10). However, these workers did not analyze the effects of the purified leukotoxin on bovine neutrophils. Other investigators reported the deleterious effects of concentrated crude leukotoxin on the oxidative burst of bovine neutrophils without observing enhancement of the oxidative burst when more dilute leukotoxin preparations were used (11, 19, 45). We believe this to be the first published report that P. haemolytica leukotoxin can directly stimulate a vigorous oxidative burst by bovine neutrophils (Fig. 2). We cannot explain why similar observations were not made in previous studies (including our own) with crude leukotoxin. Perhaps the crude leukotoxin preparations contained some other component that inhibits activation of the neutrophil oxidative burst.

The inhibitory and lethal effects of crude leukotoxin have been reported to be time and temperature dependent (14, 19, 44) and to require the presence of calcium (14, 27). These effects are thought to arise from the ability of leukotoxin to form pores (approximately 1.2 nm in diameter) in cell membranes (2, 15, 21). This results in cell swelling and calcium uptake and ultimately leads to loss of cytoplasmic constituents and cell death. Other investigators have not observed degranulation of bovine neutrophils incubated with leukotoxin without concomitant leakage of cytoplasmic markers such as lactate dehydrogenase (14, 44). These prior observations are similar to the association between release of primary granules (β-glucosaminidase activity) and lactate dehydrogenase that we observed in the present study (Table 1). It was only when we assessed the release of the secondary granule marker lactoferrin that we found evidence of selective degranulation in response to small amounts of partially purified leukotoxin (Table 1). This is similar to the selective release of secondary granules that occurs when human neutrophils are incubated with E. coli hemolysin (4). Other investigators have blocked the deleterious effects of

<table>
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<th>Leukotoxin</th>
<th>MAb</th>
<th>LDCL* (log_{10} cps)</th>
<th>Shape changeb (%)</th>
<th>Lactoferrin release* (%)</th>
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<tr>
<td>+</td>
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<td>4.65</td>
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<tr>
<td>−</td>
<td>+d</td>
<td>2.56</td>
<td>8</td>
<td>NT*</td>
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* Bovine neutrophils were stimulated with a 1:128 dilution of leukotoxin as described in the legend to Fig. 1 and in Materials and Methods. Results are the log_{10} peak counts per second (CPS) of the LDCL response of a single representative experiment.

b Bovine neutrophils were stimulated with a 1:100 dilution of leukotoxin as described in the legend to Fig. 2 and in Materials and Methods. Results are the percentage of neutrophils exhibiting shape change from a single representative experiment.

c Bovine neutrophils were stimulated with a 1:100 dilution of leukotoxin as described in the legend to Fig. 2 and in Materials and Methods. Results are the percent release of total lactoferrin (neutrophil lysate being 100%) from a single representative experiment.

dl A 1:10,000 dilution of MAb MM-601 was added to the leukotoxin before it was incubated with bovine neutrophils.

* NT, Not tested.
leukotoxin on bovine neutrophils by using antiserum from immunized calves (45), just as we prevented leukotoxin stimulation of bovine neutrophils with a neutralizing MAb (Table 2). The mechanism by which leukotoxin activates bovine neutrophils has not been identified. The uptake of calcium is likely an important event (14, 27) although there is some evidence for the role of β-adrenergic receptors in the inhibitory effects of leukotoxin on bovine neutrophils (29). Future studies will address the membrane interaction and intracellular alterations associated with the effects of P. haemolytica leukotoxin on bovine neutrophils.

We believe that we can exclude other components as being responsible for the neutrophil activation that was observed in this study. In our initial toxin preparations, P. haemolytica was grown in RPMI 1640 with lactoferrin. Although others have reported the need for an iron-binding compound for leukotoxin production (25), we obtained substantial toxin production in lactoferrin-free RPMI 1640. Comparison of lactoferrin-containing and lactoferrin-free leukotoxin preparations indicated no difference in their effects on bovine neutrophils (data not shown). We also can exclude the direct effects of lipopolysaccharide, because neutrophil activation by purified leukotoxin was heat labile, unaltered by polymyxin B, and blocked by the leukotoxin-neutralizing MAb MM-601 (Table 2). Furthermore, previous studies of the effects of lipopolysaccharide from P. haemolytica and other gram-negative bacteria on bovine neutrophils failed to achieve results as dramatic as those presented here (17).

The results of this study provide new insight into the pathogenesis of bovine pasteurellosis. It is known that neutrophils emigrate into the lung within hours after the arrival of P. haemolytica (6, 41). As these initial bacterial invaders begin to multiply in the lung, they will release small amounts of leukotoxin into the local environment. Our data suggest that these low levels of leukotoxin may stimulate nearby neutrophils to release inflammatory oxygen intermediates and granule constituents that can damage lung cells. If continued bacterial multiplication occurred, increasing amounts of leukotoxin would be released that would impair the defense functions of pulmonary neutrophils and mononuclear phagocytes (11, 19, 39). Such impairment could result in the local accumulation of lethal concentrations of leukotoxin that would kill nearby leukocytes, adding their intracellular constituents to the inflammatory milieu in the lung. The net result would be the acute fibrinous pleuropneumonia that characterizes fulminant pulmonary pasteurellosis.

ACKNOWLEDGMENTS

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