Influence of Pasteurella haemolytica A1 Crude Leukotoxin on Bovine Neutrophil Chemiluminescence

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Pasteurella haemolytica A1 crude leukotoxin (25%, vol/vol) rapidly diminished the bovine neutrophil chemiluminescence response to opsonized zymosan. This inhibition was neither prevented nor reversed by 75 mM sucrose. Dilute leukotoxin did not directly stimulate neutrophil chemiluminescence nor did it alter the chemiluminescence response of the neutrophils to opsonized zymosan.

Pasteurella haemolytica A1 causes an acute fibrinous pleuropneumonia in cattle. It is thought that neutrophils may contribute both to host defense (9) and to some of the tissue damage (4, 14) that occurs in pulmonary pasteurellosis. P. haemolytica A1 produces a potent extracellular leukotoxin that, at high concentrations, is lethal for bovine leukocytes (1, 2, 13). This leukotoxin has been shown to be part of a family of closely related exotoxins produced by various species of gram-negative bacteria (15). The alpha-hemolysin of Escherichia coli is a member of this toxin family that has been shown to have potent stimulatory effects on human granulocytes (3, 5). Although it too is lethal at high concentrations, at low concentrations it has been shown to stimulate human neutrophil chemiluminescence (5) and degranulation (3). The purpose of this study was to determine whether P. haemolytica leukotoxin has a similar modulatory effect on the oxidative activity of bovine neutrophils.

Bovine peripheral blood was obtained from healthy adult Holstein cattle, with 0.38% sodium citrate as an anticoagulant. Neutrophils were purified by using a discontinuous Percoll gradient, as described previously (9). The neutrophils were washed several times and suspended in Hanks balanced salt solution. The cell suspensions routinely were 95% neutrophils and >98% viable.

An isolate of P. haemolytica A1 isolated from a feedlot outbreak of pasteurellosis was obtained from R. Costvet (Baton Rouge, La.). To prepare crude leukotoxin, the bacteria were grown for 18 h on blood agar at 37°C. Several isolated colonies were then used to inoculate brain heart infusion broth that was incubated at 37°C for 4 h. The bacteria were harvested and suspended in RPMI 1640 tissue culture medium supplemented with bovine lactoferrin, as described by Gentry et al. (10). The medium was incubated at 37°C for 2 h. Following this, the bacteria were removed by centrifugation (6,000 × g for 15 min) and the supernatant fluid was filtered (0.45-μm pore size). Cytotoxic activity of the culture filtrate was determined with the automated colorimetric assay described by Greer and Shewen (11), by using freshly obtained bovine neutrophils as the target cells. We observed 78 and 55% cytotoxicity (four separate experiments) of neutrophils incubated for 1 h with 1:16 and 1:64 dilutions, respectively, of the leukotoxin preparation used in this study. Cytotoxicity dropped to a negligible level at dilutions of 1:256 or higher. Leukotoxin-containing culture filtrates were stored as aliquots at −70°C. Once thawed, any portion of an aliquot that was not used in the experiment was discarded.

Neutrophil oxidative activity was quantitated by luminol-dependent chemiluminescence, as described previously (9). Briefly, neutrophils (5 × 10⁶) and luminol (5 × 10⁻⁴ M) were preincubated with various dilutions of P. haemolytica leukotoxin in polystyrene tubes at 39°C in a Picolite Luminometer (Packard Instruments Co., Downers Grove, Ill.). To determine base-line chemiluminescence and chemiluminescence directly stimulated by P. haemolytica leukotoxin, reaction tubes were counted at 5-s intervals for 35 min. Following this, the neutrophils were stimulated by the addition of opsonized zymosan and chemiluminescence was recorded for an additional 35 min.

We first determined how quickly a lethal concentration (25%, vol/vol) of P. haemolytica leukotoxin would affect the opsonized zymosan-stimulated chemiluminescence response of bovine neutrophils. Figure 1 illustrates the suppression of chemiluminescence in a single experiment. A significant diminution of chemiluminescence occurred rapidly; by 60 min, the response was reduced by more than 90%, compared with control neutrophils. Figure 2 illustrates the results from six separate experiments which indicate that the incubation of neutrophils with leukotoxin for 30 min or longer significantly reduced (P < 0.05) the peak chemiluminescence response. Similar results were observed for the duration of the response (data not shown). Heat inactivation of leukotoxin (100°C for 10 min) eliminated the reduction in chemiluminescence (data not shown), suggesting that the reduction was not due to contaminating lipopolysaccharide. Control experiments indicated that unincubated RPMI 1640 with lactoferrin did not alter the neutrophil response, indicating that lactoferrin itself was not inhibitory (data not shown).

P. haemolytica leukotoxin and related toxins form transmembrane pores in the cytoplasmic membranes of cells that have bound the toxin. It was reported that the addition of hypertonic levels of sucrose to the medium reversed some of the cytotoxic effects of P. haemolytica leukotoxin on the BL3 bovine lymphoblastoid cell line (8). We were interested in determining whether hypertonic concentrations of sucrose would reverse the diminished chemiluminescence response caused by the incubation of neutrophils with leukotoxin. Our results indicate that this did not occur; incubation of bovine neutrophils with various concentrations of sucrose (0.9 to 75 mM) failed to prevent or reverse the deleterious effects of P. haemolytica leukotoxin on bovine neutrophil chemiluminescence (Table 1).

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FIG. 1. Tracing of a representative chemiluminescence response by bovine neutrophils incubated without leukotoxin (●) or with P. haemolytica crude leukotoxin (25%, vol/vol) for 30 min (▲) or 60 min (◆) before the addition of opsonized zymosan. Chemiluminescence is indicated as counts per second from a single representative experiment.

Next, we examined whether low concentrations of P. haemolytica leukotoxin would directly stimulate neutrophil chemiluminescence or alter the chemiluminescence response of neutrophils to opsonized zymosan. Table 2 demonstrates that neither event occurred. Incubation of bovine neutrophils with leukotoxin diluted serially from 1/16 to 1/16,384 did not directly elicit neutrophil chemiluminescence. Likewise, preincubation with these same dilutions of leukotoxin did not alter the chemiluminescence that ensued when opsonized zymosan was added to the neutrophils (Table 2).

The results of this study indicate that a high concentration of P. haemolytica crude leukotoxin rapidly and dramatically diminished the chemiluminescence response of bovine neutrophils stimulated with opsonized zymosan. Chang and co-workers reported similar inhibition by P. haemolytica cytotoxin of chemiluminescence by bovine neutrophils stimulated with P. haemolytica and other bacterial species (6, 7). This deleterious effect could not be prevented or reversed by making the medium hypertonic with sucrose. This is in contrast to a previous study in which leukotoxin-mediated damage to bovine BL3 lymphoblastoid cells was blocked with 75 mM sucrose (8).

Previous in vitro studies have indicated that some strains of P. haemolytica are killed by bovine neutrophils; however, the neutrophils in turn meet their demise when they are substantially outnumbered by the P. haemolytica cells (8). We (12) and others (7) have reported that P. haemolytica typically elicits a rather abrupt chemiluminescence response by bovine neutrophils, suggesting that some component of the bacteria abbreviates the oxidative burst. Our results implicate the release of leukotoxin as a likely mechanism for this impaired oxidative response. This inference is reinforced by observations in our laboratory that strains of P. haemolytica that produce large quantities of leukotoxin are poorly ingested and killed by bovine neutrophils, compared with those strains that produce modest amounts of leukotoxin (unpublished observations).

We did not detect any direct activation of chemiluminescence by incubation of neutrophils with a broad concentration range of P. haemolytica leukotoxin. This is in contrast to a previous report by Cavalieri and Snyder (5) that small amounts of the related alpha-hemolysin of E. coli stimulate a vigorous chemiluminescence response by human neutrophils. The observations of these workers were not confirmed by Bhakdi et al. (3), who observed no significant elevation in superoxide anion release by human neutrophils incubated with E. coli hemolysin. The discrepancy between these two studies is likely due to the different conditions used in the two studies.

TABLE 1. Suppression of neutrophil chemiluminescence by P. haemolytica leukotoxin is not ameliorated by the addition of sucrose to increase osmolarity

<table>
<thead>
<tr>
<th>Toxina</th>
<th>OPZb</th>
<th>Sucrose (mM)</th>
<th>Peak chemiluminescence (log10 cps)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>0.0</td>
<td>3.14</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>75</td>
<td>4.03</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>38</td>
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<td>18</td>
<td>3.19</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>9</td>
<td>3.34</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>1</td>
<td>3.38</td>
</tr>
</tbody>
</table>

*a Neutrophils were preincubated for 1 h at 37°C with crude leukotoxin (25%, vol/vol), as indicated in the text.

*a Neutrophil chemiluminescence was stimulated by the addition of opsonized zymosan (OPZ), as indicated in the text.

*a The peak chemiluminescence in counts per second is indicated for a single representative experiment.

FIG. 2. Incubation of bovine neutrophils with P. haemolytica crude leukotoxin (25%, vol/vol) rapidly diminishes their peak chemiluminescence response to opsonized zymosan. Results are expressed as the mean ± standard error of the mean log10 counts per second for six separate experiments. Asterisks indicate P < 0.05 compared with that of control neutrophils.
reports may result from differences in the production or purification of the toxin preparations used in the separate studies. To the best of our knowledge, there is no previously reported investigation of the ability of *P. haemolytica* leukotoxin to stimulate directly an oxidative burst by bovine neutrophils.

In conclusion, the results of this study suggest that the release of *P. haemolytica* leukotoxin during pulmonary pasteurellosis may compromise the ability of bovine neutrophils entering the lung to produce bactericidal oxygen intermediates. We find no evidence that the crude leukotoxin itself stimulates the release of oxygen radicals from bovine neutrophils that might contribute to the extensive lung damage that occurs in pulmonary pasteurellosis.

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**LITERATURE CITED**