Protective Role of Bovine Neutrophils in *Pasteurella haemolytica*-Mediated Endothelial Cell Damage

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The purpose of this study was to determine if *Pasteurella haemolytica* can directly injure bovine pulmonary endothelial cells (EC) and if neutrophils have a beneficial or detrimental role in bacterium-EC interaction. Various combinations of live *P. haemolytica*, heat-killed *P. haemolytica*, anti-*P. haemolytica* immune serum, polymyxin B, and bovine neutrophils were added to confluent monolayers of bovine EC. Monitoring included determination of 31Cr release from EC, phase microscopy, and transmission electron microscopy. Although toxic changes were not evident at 5 h postinoculation, both live and heat-killed *P. haemolytica* produced extensive EC damage by 22 h postinoculation. Damage by live *P. haemolytica* was prevented only when both neutrophils and immune serum were used. Polymyxin B effectively prevented the toxic effect of heat-killed *P. haemolytica*, suggesting that lipopolysaccharide was the major toxic factor. Morphological studies showed close apposition of *P. haemolytica* to EC membranes, neutrophil activation, and adherence to EC but no evidence of neutrophil-associated EC membrane damage. These studies demonstrate that neutrophils and immune serum in combination are effective in preventing EC damage mediated by live *P. haemolytica*.

*MATERIALS AND METHODS*

**Bacteria.** A field isolate of *P. haemolytica* (serotype 1, biotype A) was used in all experiments. The bacteria were grown from frozen stocks as previously described (6). Briefly, reconstituted bacteria were plated on enriched blood agar (EBA) for 18 h at 37°C, and then 7 to 10 bacterial colonies were transferred to brain heart infusion broth with 5% fetal bovine serum (FBS). After a 4.5-h incubation at 37°C, the culture was held at 4°C overnight. Preliminary studies showed that bacteria used immediately after the 37°C incubation or maintained overnight at 4°C produce similar results in EC toxicity assays. The bacterial culture was then washed once with sterile phosphate-buffered saline and the CFU per milliliter were quantitated based on *A*610. The correlation of *A*610 with CFU per milliliter was previously established in our laboratory in preliminary studies, using EBA plate colony counts of various bacterial dilutions. The CFU of the inoculum culture and also 22-h posttreatment (PT) samples per milliliter were verified by plating 10-μl aliquots of various bacterial dilutions on EBA plates overnight. Some aliquots of bacteria were heat killed by incubation in an 80°C water bath for 2 h.

**Bovine PMN.** PMN were isolated from the peripheral blood of an adult Charolais-Hereford-cross steer as previously described (31). The cell viability, determined with trypan blue, was >98%. The PMN were used immediately in experiments following isolation.

**Bovine pulmonary EC cultures.** Primary cultures of EC from the pulmonary artery were established by methods previously described (34). The cells were verified as endothelial by typical cobblestone morphology and the presence of anti-factor VIII antigen on the cell surface as described previously (32). The EC were grown in Dulbecco’s modified...
Eagle medium (DMEM; Whittaker M. A. Bioproducts, Walkersville, Md.) with 10% Rye's growth supplement (Una Ryan, University of Miami, Miami, Fla.), and experiments were conducted with DMEM and 10% FBS unless otherwise specified.

**EC cytotoxicity assay.** Toxicity of EC monolayers was determined by monitoring the release of \(^{51}\text{Cr}\) from labeled EC monolayers in 24-well tissue culture plates as previously described (21). After the addition of various bacterial variables, monolayers were incubated at 37°C on an oscillating platform. Following a 5- to 22-h incubation the \(^{51}\text{Cr}\) release by EC was determined, and the cytotoxic index (CI) was calculated based on the following formula: 
\[
CI = \frac{A - B}{C - B} \times 100,
\]
where \(A\) is sample release, \(B\) is spontaneous release, and \(C\) is total release. All variables were tested in quadruplicate, and experiments were repeated to confirm results.

**Electron microscopy of EC monolayers.** Transmission electron microscopy (TEM) morphological studies of EC monolayers were done as previously described (33). Briefly, monolayers were established on LUX coverslips in DMEM–10% FBS. Following the specified incubation period with bacterial variables, the wells were washed with Hanks balanced salt solution (HBSS), and cells were fixed with 2.0% glutaraldehyde for 60 min at 22°C and washed three times with cacodylate buffer. The coverslips were routinely processed for TEM embedding, sectioning, and staining and viewed on a Phillips 201 transmission electron microscope.

**Anti- \(P.\) haemolytica immune serum.** An adult cow was inoculated subcutaneously two times, 2 weeks apart, with a live avirulent \(P.\) haemolytica (serotype 1) vaccine (Respirvac, Beecham Laboratories, Lincoln, Nebr.). The anti-\(P.\) haemolytica serum titers 2 weeks following the last inoculation were 1/6,400 against whole bacteria, 1/3,200 against bacteria without a capsule, 1/6,400 against bacterial capsular extract, 1/3,200 against leukotoxins (culture supernatant fluid), and 1/400 against \(P.\) haemolytica endotoxin. These titers were determined by enzyme-linked immunosorbent assay techniques with the various \(P.\) haemolytica antigens as previously described (28).

**Bacterium opsonization assay.** Either live or heat-killed \(P.\) haemolytica (10⁵ CFU/ml) was suspended in media containing either 10% FBS or 10% immune serum. One-milliliter volumes of bacterial suspension were then added to PMN, mixed, and incubated at either 37 or 4°C for 2 h. By 22 h PMN had numerous pseudopodia, indicating activation (Fig. 2D).

**Statistical analysis.** The CIs for different variables were compared by using the Duncan multiple-range test to determine significant (\(P < 0.05\)) differences between groups.

**RESULTS**

**Effects of live \(P.\) haemolytica on EC.** To determine if \(P.\) haemolytica can directly damage bovine EC, we added several different concentrations of live \(P.\) haemolytica (10⁴, 10⁵, and 10⁶ CFU/ml) to confluent EC monolayers. At 2 h PT no toxic changes were evident and monolayers were 100% confluent. At 5 h PT the \(^{51}\text{Cr}\) release CI was 0 (data not shown) and no morphological changes were evident in monolayers by phase-contrast microscopy or TEM. By 22 h PT all culture wells treated with the various bacterial concentrations were uniformly overgrown with bacteria, as indicated by medium turbidity and phase microscopy. The CI was similarly high for all three bacterial concentrations, ranging from 44 to 49 (Fig. 1). Morphological changes at 22 h PT consisted of loss of EC monolayer confluency, numerous detached EC, and occasional retained elongated EC (Fig. 2B), compared with total confluency of EC in control media (Fig. 2A).

The preincubation of live \(P.\) haemolytica for 60 min at 37°C with 500 U of polymyxin B per ml protected EC from bacterium-mediated toxicity (data not shown). The polymyxin B treatment resulted in a reduction of the EC CI from 72 to 0 for 10⁴ CFU/ml, 80 to 0 for 10⁵ CFU/ml, and 68 to 8 for 10⁶ CFU/ml.

**Influence of PMN on \(P.\) haemolytica-mediated EC damage.** To determine if the toxic effect of \(P.\) haemolytica on EC could be modulated with PMN or immune serum, a dose response for live \(P.\) haemolytica was determined on confluent EC monolayers with and without the presence of PMN and/or 10% immune serum. CIs after 22 h PT are shown in Fig. 3. The immune serum by itself significantly lowered the CIs of bacterial concentrations of 10⁴ and 10⁵ CFU per well; however, optimal protection was produced by the combination of PMN and immune serum at all bacterial concentrations. PMN without immune serum did not significantly protect EC monolayers from \(P.\) haemolytica-mediated toxicity.

In the PMN-immune serum group, TEM morphological results at 2 h PT demonstrated bacteria within PMN (Fig. 2C), although some extracellular bacteria were still associated with the EC surface (Fig. 2D). PMN in both immune serum and control media groups had numerous cellular processes (pseudopodia), indicating activation (Fig. 2D). The PMN were also adherent to EC surfaces, as indicated by the close apoposition of PMN and EC and retention of PMN following TEM processing washes. By 22 h PT, all groups but the PMN-immune serum group had bacterial overgrowth (indicated by medium turbidity), less than 10% EC monolayer confluency, and only a few retained adherent cells. The EC monolayers treated with PMN and immune serum at 22 h PT were confluent, and only occasional rounded detached EC were evident. The CFU of \(P.\) haemolytica per milliliter at 22 h PT was significantly decreased in only the PMN-immune serum groups, as determined with culture well supernatant dilutions and subsequent colony counts on EBA.
FIG. 2. Bovine EC monolayers 22 h PT with control media (A; magnification, ×230), 22 h PT with live *P. haemolytica* (B; magnification, ×230), 2 h PT with live *P. haemolytica*, immune serum, and PMN (C [magnification, ×7,200] and D [magnification, ×5,040]), 22 h PT with dead *P. haemolytica* (E; magnification, ×230), and 22 h PT with dead *P. haemolytica* preincubated with 500 U of polymyxin B per ml (F; magnification, ×230). In panels C and D, note bacteria (arrows).

plates incubated overnight at 37°C. The PMN-immune serum group had 0 CFU/ml compared with $1.4 \times 10^9$ CFU for the medium control, $6.8 \times 10^8$ CFU for PMN only, and $3.6 \times 10^8$ CFU for immune serum only. The immune serum was an effective opsonin of both live and dead *P. haemolytica* as evidenced by the obvious intracellular bacteria in PMN (Fig. 2C) and quantitative bacterial opsonization data (Table 1). The presence of immune serum resulted in phagocytosis of 51 live bacteria per 200 PMN, compared with only 9 bacteria in the presence of 10% FBS. Parallel samples incubated at 4°C resulted in phagocytosis of only 1 or 2 bacteria per 200 PMN regardless of immune serum presence. The immune serum also resulted in phagocytosis of 25 heat-killed bacteria per 200 PMN versus 3 bacteria in FBS-treated PMN.

**Effects of heat-killed *P. haemolytica* on EC.** To determine if *P. haemolytica* viability was necessary to damage EC, various dilutions of heat-killed *P. haemolytica* ($10^4$, $10^6$, and $10^8$ CFU/ml) were added to confluent EC monolayers. As a positive control live *P. haemolytica* ($10^9$ CFU/ml) was included in the experiment. At 2 and 5 h PT there were no evident morphological changes in the EC monolayers; how-
ever, by 22 h variable levels of EC toxicity were evident, dependent on the concentration of dead bacteria (Table 2). The 10⁶-CFU/ml dilution produced no morphological changes and a CI of 0. The 10⁴-CFU/ml concentration resulted in a moderate loss of confluency and a CI of 14. The highest degree of toxicity was produced by 10⁵ CFU/ml, with almost complete loss of monolayer confluency (Fig. 2E) and a CI of 29. In the same experiment, live P. haemolytica produced a CI of 64 (data not shown). Simultaneous with bacterial inoculation, addition of immune serum (10%), PMN (10⁶ per well), or both did not significantly affect the CI of EC treated with either 10⁶ or 10⁹ CFU of dead bacteria per ml (Table 2). All groups had similar losses of confluency at 10⁵ CFU per well.

To determine if the toxic factor of the dead bacteria was LPS, 500 U of polymyxin B per ml was preincubated for 60 min at 37°C with heat-killed bacteria. At 2, 5, or 22 h PT there was morphological evidence of only slight EC toxicity in polymyxin B treatments (Fig. 2F). The polymyxin B lowered the 10⁴-CFU/ml from 14 to 4 and the 10⁹-CFU/ml CI from 29 to 10 (Fig. 4). The addition of PMN or immune serum in conjunction with polymyxin B resulted in decreases of the CI not significantly different than that resulting from the addition of polymyxin B alone (Fig. 4).

**FIG. 3.** Cytotoxicity based on ³¹Cr release by bovine EC 22 h after treatment with various concentrations of live *P. haemolytica* and control media (●), 10⁶ PMN per ml (○), 10% immune serum (▲) or 10⁹ PMN per ml and 10% immune serum (□). Data reflect the means and standard errors of the means. The EC CI was significantly decreased by the PMN-immune serum treatment at all bacterial concentrations and by the immune serum at bacterial concentrations of 10⁴ and 10⁹ CFU/ml (*P* < 0.05, Duncan multiple-range test).

**FIG. 4.** Cytotoxicity based on ³¹Cr release by bovine EC 22 h after treatment with various concentrations of heat-killed *P. haemolytica* and control media (●), 500 U of polymyxin B (PB) per ml (■), PB and 10⁹ PMN per ml (▲), PB, 10⁶ PMN per ml, and 10% immune serum (□), and PB and 10% immune serum (△). Data reflect the means and standard errors of the means. The EC CI was significantly decreased by all PB treatment groups, but the presence of PMN or immune serum did not significantly change the PB inhibition of cytotoxicity (*P* < 0.05, Duncan multiple-range test).

**DISCUSSION**

EC damage is an important event in the pathogenesis of bovine pulmonary pasteurellosis (6). In addition to the histological evidence of alveolar edema, fibrinous exudate, and hemorrhage (8), pulmonary alveolar septal capillary thrombosis has been observed in calves 5 h following intra-bronchial *P. haemolytica* inoculation (4b). The results of the present study clearly demonstrate that *P. haemolytica* can directly damage bovine pulmonary artery EC in vitro independent of bacterial viability, as evidenced by the EC toxicity caused by sufficient concentrations of heat-killed *P. haemolytica*. The ³¹Cr CI varied between bacterial variables and did not approach 100% toxicity even though the EC morphologically appeared completely dead, as evidenced by detachment or cellular contraction or both. Perhaps variable amounts of ³¹Cr were retained in cytoplasmic organelles or plasma membranes despite cell death. The degree of EC damage by live bacteria was approximately double that of the dead bacteria, but this difference is probably due to the greater number of bacteria that are eventually generated in live cultures (10⁵ to 10⁹ CFU/ml) versus the concentration

### TABLE 1. Comparison of opsonic properties of immune serum and FBS for *P. haemolytica*

<table>
<thead>
<tr>
<th><em>P. haemolytica</em> inoculum addition and incubation temp*</th>
<th>No. of bacteria phagocitized*&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Live</th>
<th>Heat killed (80°C, 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FBS 37°C</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10% immune serum 37°C</td>
<td>51</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>2</td>
<td>3</td>
<td></td>
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</tbody>
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*<sup>a</sup> 10⁹ CFU of bacteria per 10⁷ PMN.

### TABLE 2. Influence of immune serum and neutrophils on the toxic effects of heat-killed *P. haemolytica* on bovine EC

<table>
<thead>
<tr>
<th>Variable</th>
<th>CI* at log CFU of heat-killed <em>P. haemolytica</em>/well:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁴ 10⁶ 10⁹</td>
</tr>
<tr>
<td>10% FBS</td>
<td>0 + 0 14.3 + 4.5 29.0 + 1.9</td>
</tr>
<tr>
<td>10% immune serum</td>
<td>1.6 + 1.6 3.1 + 1.3 28.4 + 6.1</td>
</tr>
<tr>
<td>10% immune serum + PMN*</td>
<td>0 + 0 5.0 + 2.1 23.6 + 6.1</td>
</tr>
<tr>
<td>10% FBS + PMN</td>
<td>1.0 + 0.9 9.3 + 3.1 28.8 + 4.6</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Mean CI based on ³¹Cr release for eight replicates and standard error of the mean.

*<sup>b</sup> 10⁶ neutrophils per well.
of heat-killed bacteria (10^6 CFU/ml) used in our experiments. It is also possible that toxic factors responsible for EC damage may have been continuously produced by the live bacteria during active growth, while there was a static level of toxin in treatments with dead bacteria. *P. haemolytica* does produce an exotoxin, referred to as leukotoxin, that rapidly kills and lyases bovine leukocytes (1, 2, 35). However, leukotoxin does not have apparent direct toxic effects on bovine EC (6). Other, as yet unidentified, bacterial toxins may be important in EC damage, but the one factor known to directly damage EC is LPS (6, 27).

Previous studies have demonstrated that purified *P. haemolytica* LPS directly kills bovine EC (6, 27) and this toxicity can be neutralized by preincubation with polymyxin B (6). Polymyxin B binds to the lipid A moiety of LPS and effectively neutralizes many of the LPS-related biological actions (24). The effective protection of EC from dead *P. haemolytica* obtained with polymyxin B supports the important role of LPS as the chief mediator of EC damage in pulmonary pasteurellosis.

Very few studies have been reported concerning in vitro effects of live bacteria on EC cultures. Some bacteria such as *Escherichia coli* do actively bind to EC via S fimbra components (26), suggesting that bacterial adherence to EC may be important in the development of localized bacterial infections and evasion of the phagocytic clearance mechanism. A similar process may occur in the septicemic form of pulmonary pasteurellosis. Intravenous inoculation of *P. haemolytica* results in a fibrinopurulent pneumonia not unlike either the natural disease or that produced by an intratracheal bacterial inoculation (39). Although bacterial adherence was not monitored in this study, the close apposition of bacteria to EC membranes, as evidenced in transmission electron micrographs, suggest that *P. haemolytica* adherence to pulmonary EC may be an important factor in disease pathogenesis. This would enable blood-borne *P. haemolytica* to localize in the lung and establish a pneumonic focus.

Phagocyte interaction with *P. haemolytica* greatly determines the progression of natural and experimental disease. Previous studies have shown that PMN may contribute to the severity of pneumonic lesions (8, 36); however, diffuse pulmonary edema and hemorrhage still occur in the absence of circulating PMN (8). Results from the present in vitro study clearly show that PMN have a definite protective role in preventing EC damage by *P. haemolytica*. PMN in the presence of immune serum effectively phagocytized *P. haemolytica* and prevented EC damage. These results compare favorably with previous studies that showed that PMN in the proper PMN/bacterium ratio can phagocytize and kill *P. haemolytica* (14). At high bacterium/phagocyte ratios (greater than 10) bacteria reduce PMN viability, presumably because of bacterial secretion of leukotoxin. Our EC toxicity studies did not include bacterium/phagocyte ratios greater than 1 to 10 because preliminary studies demonstrated consistent bacterial overgrowth of culture wells when higher concentrations of bacteria were used.

Both PMN and immune sera were necessary in our experiments to effectively kill *P. haemolytica* and prevent EC damage. These results agree with previous studies of the necessity of *P. haemolytica* opsonization with immune serum before PMN can effectively kill the bacteria (14). In addition, our immune serum contained antileukotoxin antibodies which prevented the toxic effect of leukotoxin on PMN. Immune serum by itself, in our study, did have some protective effect against live *P. haemolytica* but not as complete as PMN and immune serum together. These results agree with previous studies which showed variable but incomplete effects of immune serum (4, 19).

It was surprising that PMN and immune serum, either singularly or in combination, did not protect EC from the toxic effect of dead *P. haemolytica*. We had postulated that in the presence of opsonizing serum, PMN would effectively phagocytize the bacteria and either degrade or absorb LPS (25). Bovine PMN contain acyloxyacyl hydroxase, which detoxifies LPS (22a). Possibly, the dead bacteria may have contained more LPS than PMN could effectively process and detoxify. This observation suggests that in the natural disease, *P. haemolytica* in adequate numbers in the lung would result in EC damage regardless of effective phagocytosis by PMN.

In this and a previous study (7), we have shown that the presence of PMN is associated with protection of the EC against either *P. haemolytica* or related products. This is contrary to previous reports which suggest that PMN are important in mediating EC damage in acute inflammatory disease (16, 17, 21, 22, 40, 41). The role of PMN in vascular damage apparently varies between in vitro culture conditions and perhaps between animal species. Our culture system contains 10% FBS, which contains components that protect EC from PMN-secreted oxygen radicals and lysosomal enzymes (41). Lack of activated PMN-mediated EC damage in the presence of serum has also recently been shown in studies with equine PMN and EC (4a.) Bovine EC are also different from human EC in that bovine EC have an effective catalase system that prevents the toxic effect of PMN-secreted hydrogen peroxide (15).

The results of the present study suggest that *P. haemolytica* can be controlled in the bovine lung provided that adequate PMN and immune serum are present and bacterial numbers are not in high concentration. However, as the numbers of bacteria increase resulting in higher LPS concentrations, EC are killed directly by the LPS or perhaps activated to a procoagulant state (3, 12, 13).

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