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# Bovine Pulmonary Endothelial Cell Damage Mediated by *Pasteurella haemolytica* Pathogenic Factors

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**The in vitro effects of *Pasteurella haemolytica* components on bovine pulmonary endothelial monolayers were investigated to determine the relative role of individual bacterial factors in the pathogenesis of bovine pulmonary pasteurellosis. Bovine pulmonary endothelial monolayers were treated with *P. haemolytica* bacterial culture supernatant (CS) and *P. haemolytica* lipopolysaccharide. At 22 h postinoculation, the CS produced severe damage to the endothelial cells, indicated by high $^{51}Cr$ release, extensive cellular detachment, and morphologic changes characterized by cell contraction, cytoplasmic blebbing, and loss of monolayer confluency. The neutralization of leukotoxin activity of the CS by heat inactivation was ineffective in decreasing the damage to endothelial cells; however, leukotoxin-neutralizing monoclonal antibody slightly diminished the toxic effect. *P. haemolytica* lipopolysaccharide by itself or as a supplement to CS produced endothelial cell damage similar to that of CS. The preincubation of CS dilutions ($10^{-1}$ and $10^{-2}$) or *P. haemolytica* lipopolysaccharide with polymyxin B almost completely eliminated cell toxicity. These studies show that *P. haemolytica* produces a soluble factor that is consistent with bacterial lipopolysaccharide and that is directly toxic to bovine pulmonary endothelial cells in vitro.**

Bovine pulmonary pasteurellosis, caused by *Pasteurella haemolytica*, is one of the most common disease problems in cattle populations. Although stress and predisposing respiratory viruses are important in initiating the pneumonic process, *P. haemolytica* infection is the major contributor to the pulmonary tissue damage observed in cattle pneumonia (6).

After colonization of the lung with *P. haemolytica*, pulmonary tissue damage occurs very rapidly. Five hours after *P. haemolytica* intrabronchial inoculation, cattle develop severe alveolar and interlobular edema, fibrinous and neutrophilic exudate, pulmonary hemorrhage, and vascular thrombosis (2, 18). These acute lesions suggest pulmonary endothelial cell damage and subsequent vascular leakage.

The *P. haemolytica* pathogenic factors that mediate this severe pulmonary tissue damage have not been well defined. A *P. haemolytica* exotoxin, referred to as leukotoxin, may be an important virulence factor due to lysis of leukocytes and release of tissue-damaging proteases (1). Although the effect of leukotoxin on nonleukocyte pulmonary cells has not been reported, leukotoxin may have a direct cytotoxic effect on pulmonary endothelial cells, resulting in the vascular damage observed in the disease.

Endotoxin or lipopolysaccharide (LPS) produced by *P. haemolytica* may also be important in the disease pathogenesis (4). It has been shown that intravenous inoculation of LPS in sheep incites acute pulmonary inflammation and endothelial cell injury (12). A similar process could occur in cattle after *P. haemolytica* pulmonary colonization and release of LPS.

The purpose of this study was to determine whether *P. haemolytica* soluble components can damage bovine pulmonary endothelial cells in vitro and to identify which bacterial factors are most important in mediating cell damage. By observing the effects of *P. haemolytica* crude leukotoxin and LPS on bovine pulmonary endothelial monolayers, we have shown that *P. haemolytica* does produce a soluble factor that is toxic to endothelial cells in vitro. This factor appears to be LPS, as evidenced by effective neutralization with polymyxin B.

## MATERIALS AND METHODS

**Endothelial cell cultures.** Primary endothelial cell cultures were established from pulmonary arteries obtained from a local abattoir by established techniques (16). Briefly, the arterial intimal surface was gently scraped with a scalpel, and the scraped cells were suspended in Dulbecco modified Eagle medium (DMEM; Whittaker MA Bioproducts, Walkersville, Md.) with 10% Ryan growth supplement (Una Ryan, University of Miami, Miami, Fla.). The cells were grown in tissue culture dishes for 24 to 48 h, and individual endothelial colonies were isolated by using glass cloning rings (Bellco Glass, Inc., Vineland, N.J.) and 0.05% trypsin (GIBCO Laboratories, Grand Island, N.Y.). The cell clones were expanded in tissue culture flasks and identified as endothelial cells by typical cobblestone morphology and the presence of anti-factor VIII antigen as described previously (15). The endothelial cells used in all experiments were of the same cell line at less than passage 20.

**Bacterial factors.** Culture supernatant from *P. haemolytica* in the log phase of growth, referred to as bacterial culture supernatant (CS), was prepared as previously described (17). Briefly, *P. haemolytica* (biotype A, serotype 1) was grown on enriched blood agar for 18 h at 37°C, followed by transfer of 7 to 10 bacterial colonies to brain heart infusion broth with 5% fetal bovine serum (FBS). After a 4.5-h incubation at 37°C, the culture was centrifuged at 10,000 × *g* for 20 min, and the supernatant was discarded. The bacterial pellet was reconstituted in RPMI 1640 medium with 10% FBS, incu-

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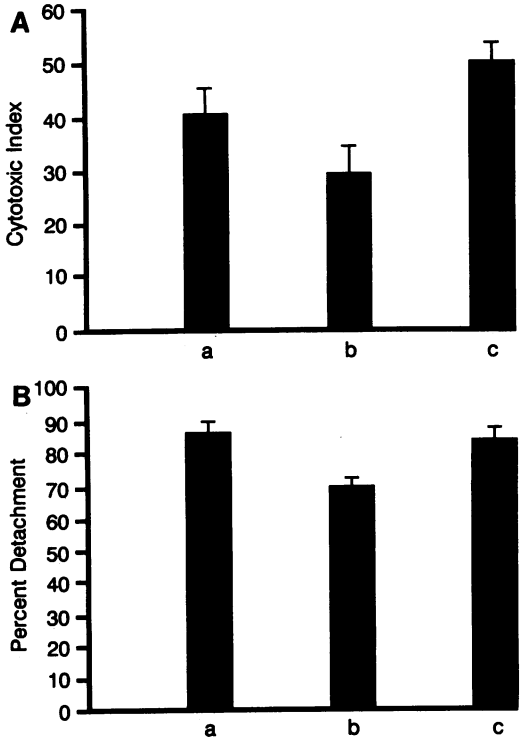


FIG. 1. Cytotoxicity (A), based on $^{51}Cr$ release and percent detachment (B), of bovine endothelial cells 22 h after treatment with a $10^{-1}$ dilution of CS (a), CS ($10^{-1}$) preincubated with anti-leukotoxin monoclonal antibody (b), and heat-inactivated CS ($10^{-1}$) (c). Data reflect the means and standard errors of the mean of two separate experiments and a minimum of eight experimental wells. There were small but significant differences between the cytotoxic indices of all three groups and a significant difference between the percent detachment of the group treated with CS (b) and the other groups ($P < 0.05$; Duncan multiple-range test).

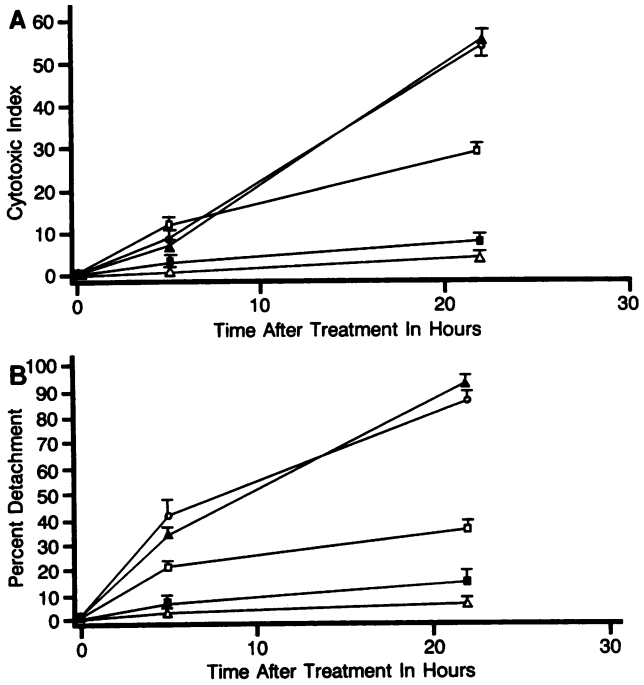


FIG. 2. Cytotoxicity (A), based on $^{51}Cr$ release, and percent detachment (B) of bovine endothelial cells 22 h after treatment with *P. haemolytica* LPS (500 ng/ml) (▲), CS (○), CS preincubated with 1,000 U of polymyxin B per ml (□), *P. haemolytica* LPS preincubated with 1,000 U of polymyxin B per ml (■), and *P. haemolytica* LPS preincubated with 1,000 U of polymyxin B per ml (△). The CS and *P. haemolytica* LPS were both significantly higher than the other three groups at 22 h after treatment ($P < 0.05$, Duncan multiple-range test). Preincubation with polymyxin B significantly decreased the CS toxic effect and almost completely eliminated the toxic effect of *P. haemolytica* LPS.

bated for 90 min at 37°C, and centrifuged at 10,000 × *g* for 20 min. The supernatant was saved and filtered through a 0.2-μm filter (Costar, Cambridge, Mass.), aliquoted, and stored at −70°C.

The leukotoxin activity of the CS was determined to be 6,855 toxic units per ml by using a previously described neutral red uptake cytotoxicity assay of bovine lymphoma cells (obtained from R. Theilen, University of California, Davis) (8). The leukocidal activity of a $10^{-1}$ dilution of CS was eliminated by either heat denaturation (56°C for 30 min) or incubation for 60 min at 37°C with leukotoxin-neutralizing monoclonal antibody. The murine monoclonal leukotoxin-neutralizing antibody was produced with conventional hybridoma monoclonal techniques. The leukotoxin-neutralizing antibody was characterized as immunoglobulin M. Ascites fluid from mice, implanted with the leukotoxin-neutralizing hybridoma clone, was used in these experiments. The neutralization of the leukotoxin with heat inactivation or monoclonal antibody was verified by the neutral red cytotoxicity assay and also by a previously described neutrophil $^{51}Cr$ release cytotoxicity assay (14).

The *P. haemolytica* LPS, extracted from bacteria by phenol-water extraction (11), was obtained from Robert Moore (Department of Microbiology, University of Tennessee, Knoxville). The endotoxin activities of the LPS preparation and CS were quantified with a chromogenic *Limulus* lysate assay (Whittaker MA Bioproducts). An *Escherichia coli* LPS standard was used to standardize the levels of endotoxin in all bacterial products. The CS contained $10^4$ endotoxin units per ml. To neutralize the endotoxic activity of the CS or *P. haemolytica* LPS, preparations were incubated for 60 min at 37°C with 1,000 U of polymyxin B (Sigma Chemical Co., St. Louis, Mo.) per ml.

**Endothelial cell cytotoxicity assay.** Endothelial cell damage was quantified by a previously described $^{51}Cr$ release assay (10). Briefly, $10^5$ cells in 1 ml of DMEM–10% RGS were plated into 24-well tissue culture plates. Upon reaching confluency in 24 h, the cells were labeled with 1 ml of $^{51}Cr$ (10 μCi/ml of DMEM–10% Ryan growth supplement; ICN Pharmaceuticals, Irvine, Calif.) for 24 h. The cells were then washed five times with Hanks buffered salt solution, and variables diluted in DMEM–10% FBS were added. The variables tested included CS ($10^{-1}$), heat-inactivated CS ($10^{-1}$), CS ($10^{-1}$) preincubated with neutralizing monoclonal antibody, CS preincubated with polymyxin B, *P. haemolytica* LPS (diluted in DMEM–10% FBS to equal the level of endotoxin units of CS), and *P. haemolytica* LPS preincubated with polymyxin B. Dose responses of endothelial cells to CS, *P. haemolytica* LPS, and *E. coli* (serotype O55:B5) LPS (Sigma) were also done. At least four wells for each variable were done in each experiment, and experiments were repeated to confirm results. At postinoculation (p.i.) times of 5 and 22 h, the plates were centrifuged, and 200 μl of media was removed and measured for $^{51}Cr$ release with a

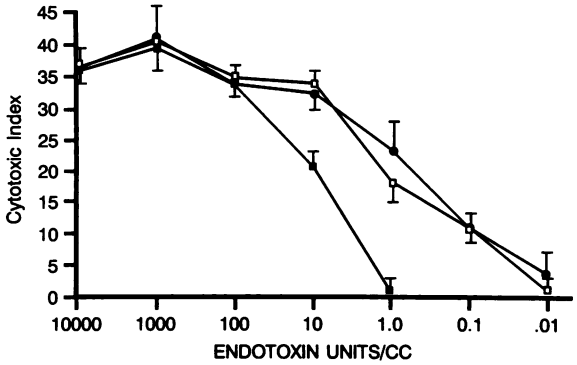


FIG. 3. Cytotoxicity, based on $^{51}Cr$ release, of bovine pulmonary artery endothelial cells 22 h after treatment with various concentrations of CS (■), *P. haemolytica* LPS (●), and *E. coli* LPS (□). The data reflect the means and standard errors of the mean for two separate experiments and a minimum of eight replicates. The CS produced significantly less cytotoxicity at the 10 and 1.0 endotoxin unit levels ($P < 0.05$, Duncan multiple-range test).

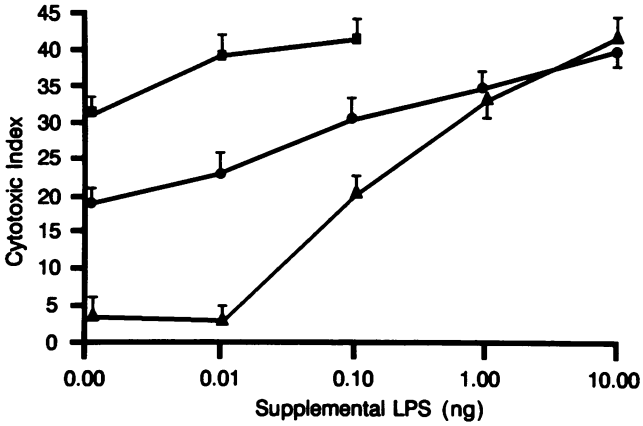


FIG. 4. Cytotoxicity, based on $^{51}Cr$ release, of bovine endothelial cells 22 h after the addition of various amounts of *P. haemolytica* LPS to $10^{-2}$ (■), $10^{-3}$ (●), and $10^{-4}$ (▲) dilutions of CS. The data reflect the means and standard errors of the mean for two separate experiments. The addition of *P. haemolytica* LPS increased the cytotoxic index until a maximum level of toxicity (38 to 42) was reached.

gamma counter. The cytotoxic index (CI) for each variable was determined using the formula $CI = [(A - B)/(C - B)] \times 100$, where $A$ is the variable release, $B$ is the spontaneous release, and $C$ is the total release.

**Cellular detachment assay.** As a more sensitive indicator of endothelial cell damage, a previously described detachment assay (19) was also used to quantify the degree of endothelial cell membrane damage and decreased adhesion to the culture well plastic surface. The experimental culture wells described above for the $^{51}Cr$ release assay were used. After removal of the 200 μl of medium for the cytotoxicity assay, the wells were washed three times with Hanks buffered salt solution and treated with 500 μl of 1% Triton X-100. The total content from each well was then measured for $^{51}Cr$. The percentage of cellular detachment was computed by the following formula: 100 × [(control − variable)/control].

**Morphological observations.** At 5 and 22 h p.i. the cell monolayers were observed with an Olympus CK2 inverted phase-contrast microscope.

**Statistical analysis.** The cytotoxic index and percent cellular detachment for different variables were compared by using the Duncan multiple-range test to determine significant differences ($P < 0.05$) between groups.

## RESULTS

**Effects of *P. haemolytica* leukotoxin on endothelial cells.** *P. haemolytica* CS (diluted $10^{-1}$ in DMEM–10% heat-inactivated fetal calf serum) produced significant damage to bovine pulmonary endothelial cells, indicated by both $^{51}Cr$ release (Fig. 1A) and detachment of cells from tissue culture wells (Fig. 1B). By 22 h p.i., the damage to cells was indicated by a cytotoxic index of 40 and a 86% detachment rate. Neutralization of the leukotoxin component of the CS by preincubation with leukotoxin-neutralizing monoclonal antibody decreased the cytotoxic index and detachment rate by a small but statistically significant amount. Heat inactivation of the CS was not effective in diminishing endothelial cell damage. The cytotoxic index of heat-inactivated leukotoxin (52 ± 3) was actually slightly higher than that of CS (40 ± 4), although the detachment rates were similar.

**Effects of *P. haemolytica* LPS on endothelial cells.** Endothelial cells incubated with either CS or extracted *P. haemolytica* LPS had similar levels of $^{51}Cr$ release (Fig. 2A) and cellular detachment (Fig. 2B). At both 5 and 22 h p.i., the CS and *P. haemolytica* LPS cytotoxic indices were almost identical. Although there was some variation between the detachment rate of the two groups, the differences were not significant ($P < 0.05$). The dose responses of *P. haemolytica* LPS, *E. coli* LPS, and CS are shown in Fig. 3. The cytotoxic index was similar for all three preparations until the levels of 10 and 1 endotoxin units, when the crude leukotoxin cytotoxic index was significantly less than those of *E. coli* and *P. haemolytica* endotoxins. There was approximately a 100-fold difference in the endpoint titration of CS versus that of *P. haemolytica* and *E. coli* LPS.

The addition of various amounts of *P. haemolytica* LPS to

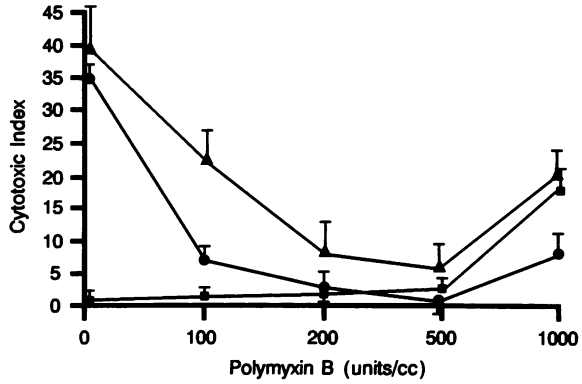


FIG. 5. Cytotoxicity, based on $^{51}Cr$ release, of bovine endothelial cells 22 h after the addition of $10^{-1}$ (▲) and $10^{-2}$ (●) dilutions of *P. haemolytica* culture supernatants preincubated 60 min with polymyxin B. A polymyxin B control without culture supernatant is also shown (■). The addition of polymyxin of B produced significant decreases in toxicity to an optimal level of 500 U of polymyxin B per ml. Levels of 1,000 U of polymyxin B per ml enhanced cytotoxicity in all three treatment groups. The data represent means and standard errors of the mean of two experiments and a minimum of eight experimental wells.

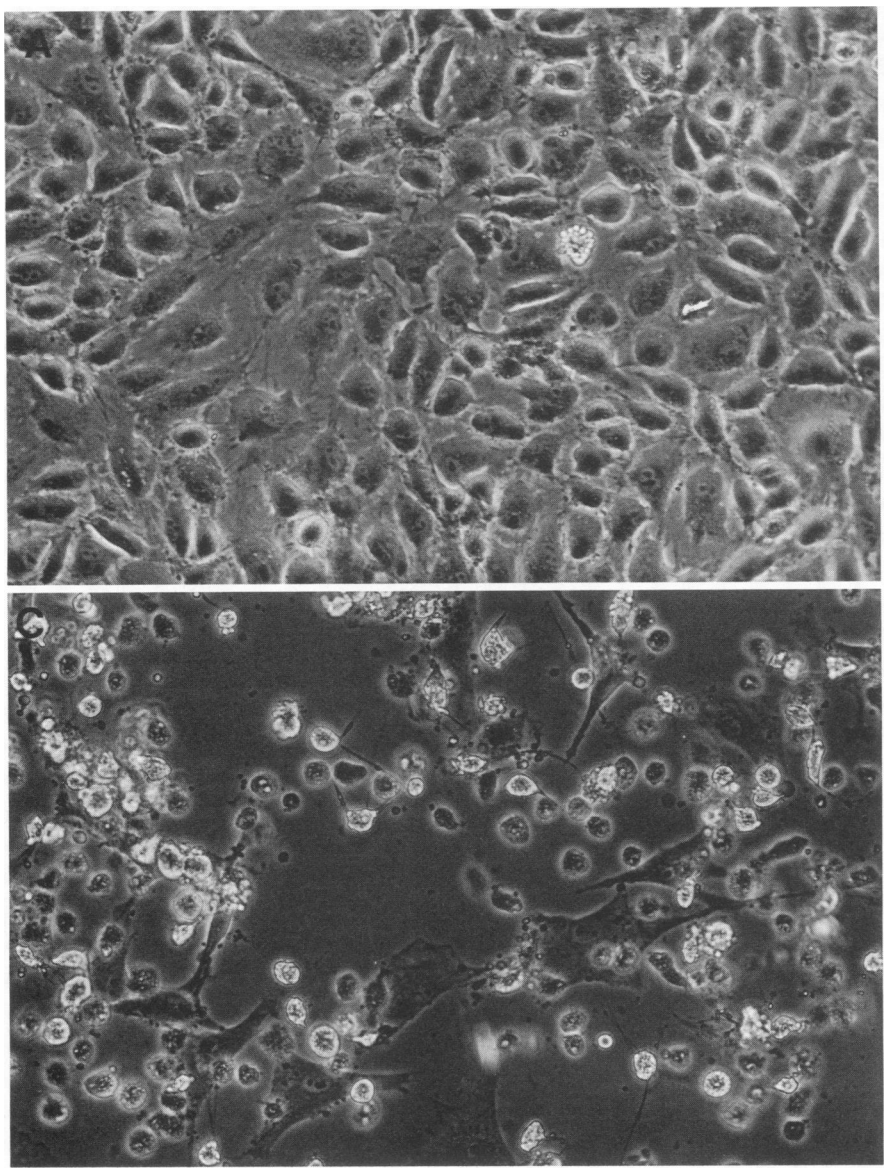

FIG. 6. Bovine pulmonary artery endothelial cell monolayer before treatment (A). Bovine endothelial cells at 5 h (B) and 22 h (C) after treatment with *P. haemolytica* CS and 22 h after treatment with CS preincubated with 1,000 U of polymyxin B per ml (D). Note the occasional rounded, contracted, degenerative cells with cytoplasmic blebbing in panel B and the lack of confluency, numerous rounded degenerative cells, and occasional elongated attached cells in panel C. Treatment of CS with polymyxin B (D) resulted in only occasional rounded cells and retention of monolayer confluency. (Magnification, ×132)

the culture supernatant increased damage to endothelial cells (Fig. 4). When 0.01 ng of LPS was added to dilutions of CS the cytotoxic index increased in the $10^{-2}$ and $10^{-3}$ CS dilutions but not in the $10^{-4}$ dilution. However, the addition of 0.10 ng of LPS elevated the cytocidal index in all three groups, demonstrating that the LPS component of CS was important in mediating endothelial cell injury. A maximum cytocidal index of 39 to 42 was achieved with the addition of 0.10 ng to the $10^{-2}$ CS dilution and 10.0 ng to the $10^{-3}$ and $10^{-4}$ CS dilutions.

Attempts to prevent endothelial cell damage by binding the lipid A portion of the LPS with polymyxin B were variably successful, depending on the concentrations of CS and polymyxin B. Undiluted CS preincubated with 1,000 U of polymyxin B per ml produced cytotoxic indices of 12 at 5 h and 29 at 22 h p.i. (Fig. 2A). The detachment rate was also significantly decreased in the CS groups treated with polymyxin B (Fig. 2B). The preincubation of *P. haemolytica* LPS with polymyxin B almost completely eliminated cell damage, with a cytotoxic index of only 3 and a detachment rate of only 5% at 22 h p.i.

More complete neutralization of the LPS component of the CS was attempted with dilutions of CS and various concentrations of polymyxin B (Fig. 5). The addition of 100, 200, and 500 U of polymyxin B per ml to either $10^{-1}$ or $10^{-2}$ dilutions of CS produced significant decreases in cytotoxic indices. The culture supernatant dilution of $10^{-2}$ produced a cytotoxic index of only 2 after preincubation with 500 U of polymyxin B per ml. However, the addition of 1,000 U of polymyxin B actually raised the cytotoxic index in all

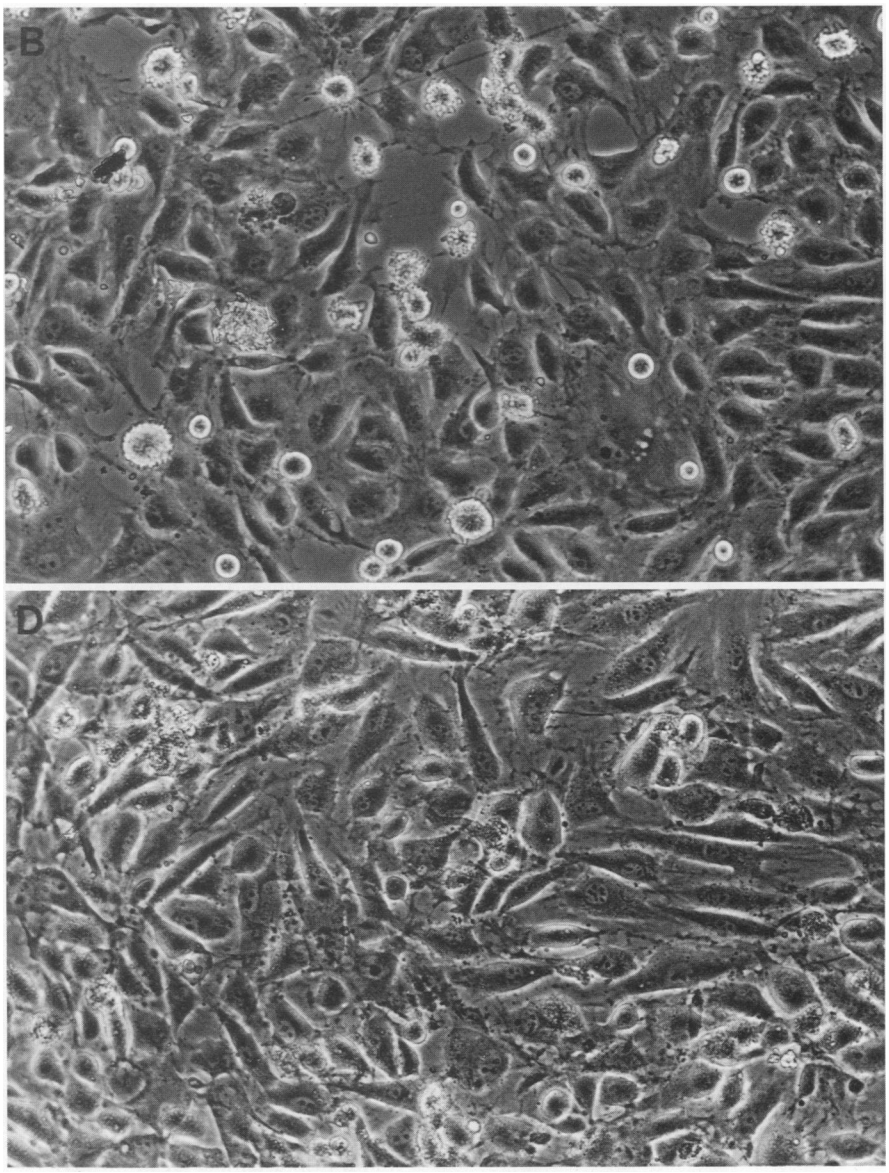


FIG. 6—*Continued*

variable groups, compared with those with 500 U, presumably due to the toxic effects of excessive polymyxin B as evidence by cytotoxic indices in the absence of CS.

**Morphological changes in endothelial monolayers.** In addition to the $^{51}Cr$ release and cell detachment, there were corresponding morphological changes in the endothelial cell monolayers compared with control endothelial cells (Fig. 6A). By 5 h p.i., several degenerative changes were evident in monolayers treated with CS, heat-inactivated CS, CS with leukotoxin-neutralizing monoclonal antibody, and *P. haemolytica* LPS. These changes were characterized by cell contracture, partial loss of monolayer confluency, and detachment of occasional cells (Fig. 6B). By 22 h p.i., there were more severe degenerative changes consisting of almost complete loss of confluency, numerous detached shrunken cells, cytoplasmic blebbing, and distortion of remaining attached cells (Fig. 6C).

The preincubation of crude leukotoxin with polymyxin B prevented some but not all of the cellular degenerative changes. By 22 h p.i., approximately 80 to 90% of the cells were still attached, although occasional cells were contracted and detached (Fig. 6D). Preincubation of *P. haemolytica* LPS with polymyxin B was successful in preventing any evident morphologic changes in the endothelial monolayers.

## DISCUSSION

The results from these studies demonstrate that soluble factors produced by *P. haemolytica* have a severe damaging effect on bovine endothelial cells in vitro. A factor in the *P. haemolytica* culture supernatant responsible for mediating endothelial cell toxicity appears to be the LPS constituent. This is evidenced by similar cell toxicity by both *P. haemolytica* LPS and CS. The addition of LPS to CS dilutions also resulted in increased endothelial cell damage, corre-

sponding to the amount of LPS added. Also, the protective effect of polymyxin B further suggests that the endotoxin component of the bacterial culture supernatant is predominantly responsible for the endothelial cell toxicity. Initial studies with 1,000 U of polymyxin B per ml suggested that the undiluted CS toxic effect could not be completely neutralized. However, further studies demonstrated that, by adjusting the concentration of CS and polymyxin B, endothelial cells were almost completely protected from CS toxicity. The use of a high (1,000 U/ml) concentration of polymyxin B actually resulted in increased endothelial cell toxicity, presumably due to a direct toxic effect of the polymyxin B itself.

The effects of *P. haemolytica* endotoxin on endothelial cells have not been well investigated; however, *E. coli* endotoxin directly damages bovine endothelial cells (7, 9, 13). Similar to our results, endothelial cytotoxicity due to *E. coli* endotoxin is not a rapid change, and 5 to 18 h of incubation is necessary for detectable cytotoxic effects. The morphological changes of cell retraction, cellular protoplasmic blebbing, and cell detachment reported in these previous studies are similar to what we observed in our experiments.

Our results demonstrate that the leukotoxin component of *P. haemolytica* culture supernatant is not the major component that directly damages endothelial cells. The inactivation of the heat-labile leukotoxin by heat denaturation actually slightly increased the cytotoxic index; however, the detachment rates were similar for both CS and heat-inactivated CS. The preincubation of CS with leukotoxin-neutralizing monoclonal antibody had only a small effect in diminishing the degree of endothelial cell toxicity. This reduction of toxicity by monoclonal antibody may be due to nonspecific interaction of LPS (present in CS) with mouse ascitic fluid, because other nonspecific monoclonal antibodies produce a similar slight decrease of toxicity (Breider, unpublished data). The importance of leukotoxin in the pathogenesis of pulmonary pasteurellosis should not be completely dismissed, because leukotoxin can effectively kill and lyse bovine leukocytes (1), possibly leading to secondary endothelial cell damage (17).

The mechanism of direct endotoxin-mediated endothelial cell toxicity is not well defined. Endotoxin may activate the endothelial cells to produce reactive oxygen species such as superoxide radicals, hydrogen peroxide, or hydroxyl radicals, leading to cell membrane damage (9). In support of this hypothesis, the addition of an oxygen radical scavenger, dimethyl sulfoxide, can prevent endotoxin-mediated damage to endothelial cells in vitro (3).

In addition to directly damaging bovine endothelial cells, endotoxin may promote pulmonary tissue damage by stimulating an excessive inflammatory response. Endotoxin can induce macrophages in vitro to produce two potent proinflammatory cytokines, interleukin-1 and tumor necrosis factor (5). The interaction of these cytokines with neutrophils, platelets, and endothelial cells can induce localized hemorrhage, edema, and thrombosis in rabbit models of endotoxemia (5). A similar mechanism of tissue damage may be present in bovine pulmonary pasteurellosis. Endotoxin from intrapulmonary *P. haemolytica* may cause direct endothelial damage and also initiate interleukin-1 and tumor necrosis factor production by alveolar macrophages, leading to a procoagulant state in the lung. This progression of events could explain the severe vascular thrombosis, hemorrhage, edema, and tissue necrosis usually present in pulmonary pasteurellosis.

To further completely understand the mechanisms leading to *P. haemolytica*-induced lung injury, other components of the host response need to be investigated. However, the direct toxic effect of *P. haemolytica* endotoxin on pulmonary endothelial cells appears to be an important factor in pulmonary tissue damage induced by bacteria.

## ACKNOWLEDGMENTS

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