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Enhancement of Neutrophil-Mediated Injury to Bovine Pulmonary Endothelial Cells by *Pasteurella haemolytica* Leukotoxin

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In this study, we used an in vitro coculture system to determine which virulence factor from *Pasteurella haemolytica* A1 was responsible for augmenting bovine polymorphonuclear neutrophil (PMN)-mediated killing of bovine pulmonary artery endothelial cells (BPAEC). A ^{51}Cr release cytotoxicity assay was used as a measure of BPAEC killing. The mechanisms associated with this BPAEC killing were also studied. Our results demonstrated that the leukotoxin and not the lipopolysaccharide from *P. haemolytica* was responsible for augmenting the PMN-mediated killing of BPAEC. Furthermore, this augmented killing was related to the stimulation of PMNs by the leukotoxin. Killing of BPAEC by leukotoxin-stimulated PMNs was diminished in the presence of the H_2O_2 inactivator, catalase. The membrane-permeant H_2O_2, hydroxyl radical (HO·) scavenger 1,3-dimethyl-2 thiourea, and the HO· scavenger dimethyl sulfoxide but not the myeloperoxidase inhibitor sodium azide attenuated this BPAEC killing. Pretreatment of BPAEC with a 21-aminosteroid (U74500A), a potent iron chelator-antioxidant, provided the most effective protection against BPAEC killing induced by leukotoxin-stimulated PMNs. These data were compatible with the concept that the H_2O_2 generated by leukotoxin-stimulated PMNs interacts with intracellular iron in the endothelial cell to form highly reactive HO·. We suggest that HO· may be a key factor in BPAEC killing. Furthermore, since the elastase-specific inhibitor *N*-methoxy-succinyl-Ala-Ala-Pro-Val-chloromethyl ketone (CMK) also attenuated BPAEC killing and both CMK and 1,3-dimethyl-2 thiourea functioned additively in protecting against BPAEC killing, we conclude that both HO· and elastase may jointly contribute to BPAEC killing induced by leukotoxin-stimulated PMNs. This study broadens our understanding of how leukotoxin-stimulated PMNs injure lung endothelial cells and provides new insight into the pathogenesis of bovine pneumonic pasteurellosis.

Bovine pneumonic pasteurellosis is the most important respiratory disease of cattle in Western Europe and North America both clinically and economically (1, 48). The disease is multifactorial in nature, involving infection by a variety of microorganisms in conjunction with stressful management practices and environmental factors. *Pasteurella haemolytica* biotype A, serotype 1 (A1), has been established as the primary agent responsible for the clinical disease and the pathophysiologic events characterized by acute lobar fibrinonecrotizing pneumonia (12, 31). Interestingly, notable similarities exist between the pathophysiologic alterations seen in this disease and human adult respiratory distress syndrome (42).

Experimental pneumonic pasteurellosis can be induced in cattle by either transthoracic, intrapulmonic, or intratracheal inoculation of logarithmic-phase *P. haemolytica* A1 organisms (2, 26). We (46, 47) and others (23, 33, 39) have characterized the progression of the microscopic and ultrastructural pneumonic lesions that occur in natural and experimentally induced pneumonic pasteurellosis. These studies suggest that early in the infection, an influx of neutrophils into the alveoli occurs, followed by accumulation of extensive edema fluid containing fibrin in the alveoli, pleural surface, and interlobular septa. Damage to the pulmonary endothelium is observed beneath the sites of neutrophil attachment, suggesting that neutrophils play a key role in the vascular leakage and the ensuing alveolar edema. Other lesions which appear later include hemorrhaging, vascular thrombosis, coagulative parenchymal necrosis, and abscess formation. The underlying mechanisms leading to lung damage remain poorly understood.

P. haemolytica A1 produces several potential virulence factors, including lipopolysaccharide (LPS) with endotoxin properties, an exotoxic leukotoxin, capsular material, and fimbria (9). A growing body of evidence supports the contention that all of these factors are involved in the pathogenesis of the disease. Of these, the leukotoxin and LPS have received the most attention. We have demonstrated previously (45) the presence of leukotoxin and LPS in situ in acute lung lesions induced by *P. haemolytica* in cattle, suggesting an important role for these in the pathogenesis of the disease. Leukotoxin is a pore-forming cytolysin and is a member of the genetically related family of toxins termed repeats in toxin (RTX). Toxins in this family include the hemolysin of *Escherichia coli*, the leukotoxin of *Actinobacillus* species, and the bifunctional adenyl cyclase hemolysin of *Bordetella pertussis* (44). The leukotoxin of *P. haemolytica* is distinguished from other RTX toxins by its narrow target cell specificity, being cytocidal only to ruminant leukocytes and platelets (21). Other in vitro studies (11, 25) have shown that the leukotoxin, in addition to its cytocidal effect on bovine neutrophils, also stimulates a respiratory burst and degranulation of lysosomes. Stimulation of the respiratory burst, as evidenced by generation of toxic oxy-

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gen radicals, is an immediate event, while cytolysis and release of proteases by degranulation occur over a longer period of time (25).

Several studies have also shown that the LPS from *P. haemolytica* is a major virulence factor involved in the induction of lung injury. It can directly activate (28) and also damage (7, 29) bovine pulmonary endothelial cells. Additionally, it can increase adherence of neutrophils to the endothelial cells (28) and modulate neutrophil function (10). Furthermore, intratracheal or intravenous inoculation of *P. haemolytica* LPS leads to pathophysiologic derangements in the calf lung (34).

The pulmonary endothelium is a critical component of the barrier between the vascular space and the alveoli. It functions as a permeability barrier and has other important metabolic functions related to blood flow and blood clotting. Neutrophil adherence to pulmonary endothelium and the ensuing endothelial cell injury which is responsible for the vascular leakage are important events in the pathogenesis of pneumonic pasteurellosis (46, 47). It is unclear which virulence factors from *P. haemolytica* contribute to this neutrophil-mediated endothelial cell injury. In a study using an in vitro neutrophil-endothelial cell coculture system, Breider et al. (8) attributed endothelial cell injury to the killing of neutrophils by *P. haemolytica* leukotoxin. The purpose of the present study was to identify which virulence factor from *P. haemolytica* was responsible for augmenting the neutrophil-mediated endothelial cell injury and to examine the mechanisms potentially involved in this injury.

(Parts of this work have been published as an abstract [24].)

MATERIALS AND METHODS

Chemicals and reagents. Phorbol myristate acetate (PMA), catalase, soybean trypsin inhibitor (SBTI), pentoxifylline, *N*-methoxy-succinyl-Ala-Ala-Pro-Val-chloromethyl ketone (CMK), dimethyl sulfoxide (DMSO), and sodium azide were purchased from Sigma Chemical Co. (St. Louis, Mo.). Fetal bovine serum was purchased from Irvine Scientific (Santa Ana, Calif.); Dulbecco's modified Eagle medium (DMEM) was purchased from GIBCO (Grand Island, N.Y.); RPMI 1640 medium was purchased from Whittaker Bioproducts, Inc. (Walkersville, Md.); 1,3-dimethyl-2 thiourea (DMTU) was purchased from Aldrich Chemical Co. (Milwaukee, Wis.); polymyxin B sulfate (5,000 U/mg) was purchased from Wako Chemicals, Inc. (Richmond, Va.), and the 21-aminosteroids U74500A and U74389F were provided by David Zimmermann (Upjohn Co., Kalamazoo, Mich.). A stock solution of PMA (1 mg/ml) in DMSO was stored frozen at −70°C and diluted in RPMI 1640 supplemented with 0.5% bovine serum albumin (BSA) at the time of use. U74500A and U74389F were freshly dissolved in DMSO, and a 4 mM solution of each compound was prepared in distilled water, adjusted to pH 3.0, and kept at 4°C. These stock solutions were diluted to the desired final concentration with DMEM and used the day they were prepared.

Preparation of leukotoxin-containing *P. haemolytica* culture supernatant. Leukotoxin was prepared by growing *P. haemolytica* A1 strain 12296 in RPMI 1640 tissue culture medium and collecting the logarithmic-growth-phase culture supernatant as described by Penaredondo et al. (30). The culture supernatant was separated from the bacteria by centrifugation and filter sterilized (0.45-μm pore size). The supernatant was then concentrated fivefold by means of a spiral fiber apparatus (model CH2S; Amicon Corp., Danvers, Mass.) equipped with a spiral cartridge (model S11Y30) and dialyzed against pyrogen-free distilled water by using the dialyzing mode of the same apparatus. The retentate was lyophilized and stored in a desiccator at 4°C. This culture supernatant (henceforth referred to as crude leukotoxin) contained a mixture of leukotoxin, LPS, capsular polysaccharide (CP), and few unknown proteins (45). The leukotoxic activity was quantified by a previously described colorimetric assay using the BL-3 bovine lymphoma cell line obtained from R. Thielen (University of California, Davis) and expressed in leukotoxin units (38). The protein concentration in the crude leukotoxin was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.). The endotoxin present in the crude leukotoxin preparation was quantified with a chromogenic *Limulus* amebocyte lysate assay obtained from Whittaker Bioproducts. The crude leukotoxin preparation utilized in our studies contained 750 U/mg (dry weight), 58 μg of protein per mg, and 5.5×10^4 endotoxin units per mg. The same leukotoxin preparation was used in all experiments.

MAbs. The murine leukotoxin-neutralizing monoclonal antibody (MAb) MAb-601 was kindly provided by S. Srikumaran, University of Nebraska, Lincoln. Ascitic fluid containing MAb-601 had a leukotoxin neutralization titer of 1:40,960 (38). MAb-601 was characterized as immunoglobulin G1 (15) and was depleted of leukotoxin-neutralizing activity by passage through protein G-agarose (Schleicher & Schuell, Inc., Keene, N.H.). MAb-1H3, which was generated and characterized in our laboratory, was directed against an epitope in the core oligosaccharide region (but not the lipid A portion) of the LPS from *P. haemolytica* and was of the immunoglobulin G3 isotype (30). This MAb reacted serologically with the LPS but did not neutralize its biological activity. The MAb designated MAb-11B6 reacted with the CP of *P. haemolytica* and was of the immunoglobulin M isotype (30). Ascitic fluids containing MAb-601, MAb-1H3, and MAb-11B6 were used in these experiments. Control mouse ascitic fluid produced in BALB/c mice implanted with the NS-1 myeloma cell line was purchased from Sigma.

Preparation of bovine neutrophils. Healthy Holstein cows over 3 years of age served as blood donors for these studies. Polymorphonuclear neutrophils (PMNs) were isolated from peripheral blood as described previously (43). Briefly, the blood was centrifuged at 700 × *g* for 1 h over a discontinuous Percoll (Sigma) density gradient. The Percoll and water used for hypotonic lysis were autoclaved for 90 min to destroy LPS contaminants. The granulocytes were pelleted with the erythrocytes, and the erythrocytes were hypotonically lysed. A second or third hypotonic lysis of residual erythrocytes was done as needed. The granulocyte cell suspension was diluted to the desired concentration in RPMI 1640 supplemented with 0.5% BSA, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The purity of the neutrophils varied from 80 to 98%, with eosinophils, not mononuclear cells, as major contaminants. Only cell suspensions containing >95% neutrophils were used in these studies. Viability, as determined by the trypan blue exclusion test, was >98%.

Culture of endothelial cells. Primary bovine pulmonary artery endothelial cells (BPAEC) were established from pulmonary arteries of 4- to 5-month-old fetuses by a modification of a previously described technique (37). Briefly, under aseptic conditions, the intimal surface of the pulmonary artery was gently scraped with a sterile Pasteur pipette containing DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin, 100 U/ml, and

streptomycin, 100 μg/ml). Endothelial cell clusters were dispersed by aspiration through a Pasteur pipette and then transferred to several 25-cm^2 tissue culture flasks (Becton Dickinson and Co., Lincoln Park, N.J.). An additional 5 ml of cell culture medium was added to each flask before incubation for 24 h at 37°C in the presence of 5% CO_2. The medium was changed after this period, and the flasks were incubated for an additional 5 to 7 days. Cell growth in the flasks was microscopically examined, and endothelial cells were identified by their typical cobblestone appearance. They were confirmed as endothelial cells on the basis of reactivity with antibodies to factor VIII-related antigen (32). The endothelial cells were then expanded in 75-cm^2 flasks. When the primary cultures became confluent, BPAEC were detached with a trypsin-EDTA solution (GIBCO; 0.05% trypsin plus 0.53 mM EDTA) diluted 1:5 in sterile saline. The suspended BPAEC were pelleted at 1,000 × *g* for 10 min at 4°C. They were resuspended in freezing medium (DMEM containing 30% fetal bovine serum and 8% DMSO), divided into 1-ml aliquots, and stored in liquid N_2 until needed. All experiments were done with BPAEC passaged between two to seven times and within 24 h of reaching confluence.

Endothelial cell injury assay. Neutrophil-mediated endothelial cell injury was measured by a standard ^{51}Cr release assay and performed essentially as described previously (37) with minor modifications. Briefly, BPAEC were seeded into 48-well tissue culture plates (GIBCO) and cultured in DMEM containing 10% fetal bovine serum and antibiotics until the cells reached confluency (approximately 2×10^5 to 5×10^5 BPAEC per well). The wells were washed once with Hanks balanced salt solution (HBSS; GIBCO), incubated with 150 μl of HBSS containing 2 μCi of ^{51}Cr-sodium chromate (Amersham, Arlington Heights, Ill.) for 3 h at 37°C in 5% CO_2, and finally washed three to five times with HBSS before use. Thereafter, bovine PMNs were added in a volume of 300 μl of RPMI 1640 supplemented with 0.5% BSA and antibiotics to BPAEC monolayers (hereafter referred to as the PMN-BPAEC coculture system). The effector (PMN)-to-target cell (BPAEC) ratio was always 5:1. The PMNs were allowed to settle onto the BPAEC monolayer for 30 min at 37°C in 5% CO_2 before addition of the stimulants (100 μl of PMA or crude leukotoxin) since evidence from our laboratory and others (34) suggested that close proximity between PMNs and target cells was required for optimal interaction. When inhibitors were used, they were added to the PMN suspension with few exceptions. In the experiment with polymyxin B, crude leukotoxin was preincubated for 60 min at 4°C with various concentrations of polymyxin (10 to 120 μg/ml), and 100 μl of the mixture was added to the PMN-BPAEC coculture system. When the 21-aminosteroid drugs U74500A and U74389F were used as inhibitors, they were preincubated with BPAEC for 10 h and the unincorporated drug was washed off before addition of PMNs. As reported by others, this step was necessary to allow sufficient time for the 21-aminosteroids to enter the BPAEC (3). After incubation of the cocultures for 18 h at 37°C in 5% CO_2, the medium fluid (0.5 ml) was removed from each well and centrifuged and a 125-μl aliquot from each well was measured for ^{51}Cr release with a gamma counter. The percentage of cytotoxicity was calculated by using the formula $[(A - B)/(C - B)] \times 100$, where A was experimental release, B was spontaneous release, and C was the total release. Each variable was tested in quadruplicate, and each experiment was repeated three to six separate times.

Statistical analysis. All results were expressed as the mean ± standard deviation (SD). One-way analysis of variance and Duncan's multiple-range tests were used to determine statistical significant differences between groups. The term "significant" in this article indicates probability of less than 0.05.

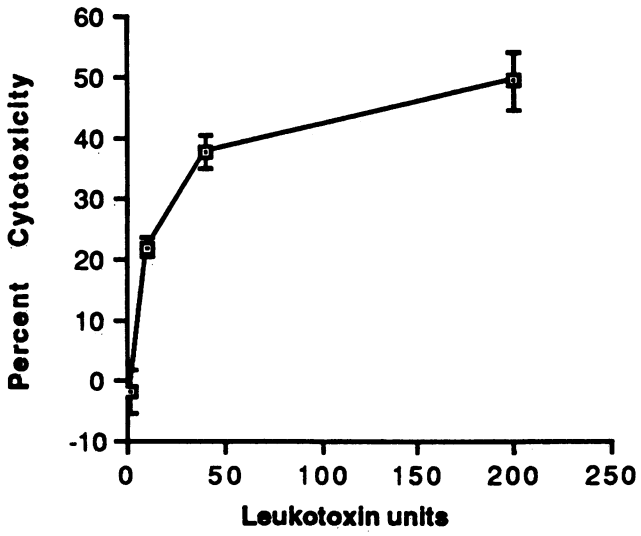

FIG. 1. Dose-dependent enhancement of PMN-mediated BPAEC killing by crude leukotoxin from *P. haemolytica*. ^{51}Cr-labeled BPAEC monolayers in 48-well tissue culture plates were incubated for 30 min with bovine PMNs at a PMN-to-BPAEC ratio of 5:1. The PMN-BPAEC cocultures were exposed to different concentrations (1 to 200 U) of crude leukotoxin and incubated for 18 h at 37°C. At the end of incubation, the medium fluid was removed from each well and centrifuged and the percentage of BPAEC cytotoxicity was calculated as described in the text. Each datum point represents the mean ± SD based on quadruplicate samples in a single experiment that is representative of three.

RESULTS

Effects of leukotoxin on neutrophil-mediated injury to endothelial cells. To assess whether crude leukotoxin from *P. haemolytica* augmented PMN-mediated killing of BPAEC, various concentrations of crude leukotoxin were incubated with PMN-BPAEC cocultures or BPAEC alone and the ^{51}Cr release cytotoxicity assay was used as a measure of BPAEC killing. In companion studies, conditions for killing of BPAEC by PMA-stimulated PMNs were optimized. Crude leukotoxin, at concentrations from 1 to 200 U, had a marked concentration-dependent killing of BPAEC in the presence of PMNs (Fig. 1). However, in the absence of PMNs, crude leukotoxin at the same concentrations caused minimal killing of BPAEC (1.1% ± 3.0% to 5.5% ± 1.7% cytotoxicity). This increase in PMN-mediated BPAEC killing induced by crude leukotoxin was statistically significant. Likewise, the killing of BPAEC by PMA-stimulated PMNs was also concentration dependent (four experiments). Maximal killing of BPAEC occurred at a PMA concentration of 10 ng/ml. Higher PMA concentrations failed to show any significant increase of BPAEC killing. Factors such as PMNs from different cows and day-to-day handling variables contributed to variations in the magnitude of the percentage of BPAEC cytotoxicity in each experiment. Similar functional variability of PMNs from different cows has been reported previously by others (20). However, the relationship between

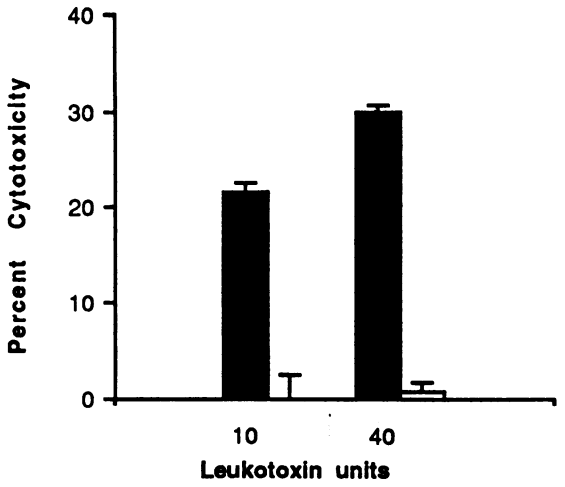

FIG. 2. Comparison of BPAEC killing induced by either crude leukotoxin-stimulated PMNs or PMNs stimulated with heat-inactivated crude leukotoxin. The PMN-BPAEC cocultures were incubated with two different concentrations (10 or 40 U) of crude leukotoxin (■) or its heat-inactivated (121°C for 30 min) product (□) for 18 h at 37°C. Percent BPAEC cytotoxicity was determined as described in the text. Bars indicate mean values from quadruplicate samples in a single experiment, and error bars indicate SDs. The experiment was repeated three times with similar results.

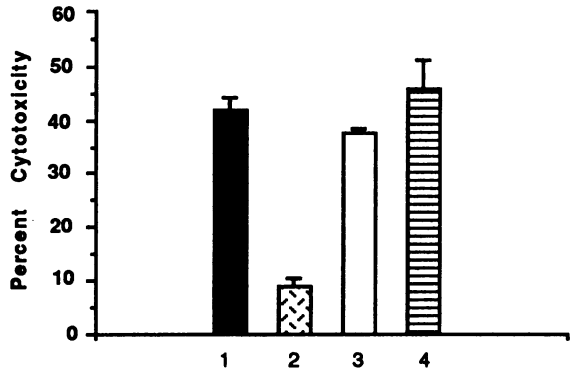

FIG. 3. Inhibition of BPAEC killing induced by crude leukotoxin-stimulated PMNs by leukotoxin-neutralizing MAb. PMNs were mixed with a 1:5,000 dilution (bar 2) of leukotoxin-neutralizing MAb-601, a 1:5,000 dilution (bar 3) of anti-LPS MAb-1H3, or a 1:5,000 dilution (bar 4) of normal BALB/c mouse ascitic fluid and added to ^{51}Cr-labeled BPAEC monolayers. After a 30-min incubation, the cocultures were exposed to crude leukotoxin (40 U) and incubated for 18 h at 37°C. Percent BPAEC cytotoxicity was calculated as described in the text. Bar 1 represents BPAEC killing induced by crude leukotoxin-stimulated PMNs in the absence of MAb. Data reflect the means ± SDs from quadruplicate samples in a single experiment. The same experiment was carried out three times with similar results.

PMA or crude leukotoxin concentration and the percentage of BPAEC cytotoxicity was similar for each experiment.

Since the crude leukotoxin preparation contained LPS and CP as contaminants, it was necessary to establish that the augmentation of PMN-mediated BPAEC killing seen in these studies was brought about by leukotoxin and not by LPS or CP. Heating of crude leukotoxin at 121°C for 30 min, which destroyed cytocidal properties of the leukotoxin but retained CP (30) and LPS with endotoxic activity, completely abrogated the augmentation of PMN-mediated BPAEC killing (Fig. 2). The leukotoxin-neutralizing MAb-601 dose dependently inhibited BPAEC killing by crude leukotoxin-stimulated PMNs (Fig. 3). MAb-1H3 that was directed against the core oligosaccharide region of the LPS of *P. haemolytica* did not inhibit BPAEC killing (Fig. 3). Polymyxin B, which neutralized the endotoxic activity in crude leukotoxin at concentrations of 30 μg/ml and higher, had no inhibitory effect on BPAEC killing (four experiments). Furthermore, the addition of MAb-11B6 which reacted with the CP of *P. haemolytica* or control ascitic fluid failed to inhibit BPAEC killing (three experiments). MAb-601 depleted of leukotoxin-neutralizing activity by passage through protein G-agarose or the addition of an irrelevent, isotype-matched MAb had no inhibitory effect on BPAEC killing by crude leukotoxin-stimulated PMNs (on the basis of three separate experiments). Furthermore, MAb-601 alone was not cytotoxic to BPAEC.

Modulation of neutrophil-mediated injury to endothelial cells with various inhibitors. We evaluated the ability of various inhibitors to protect BPAEC from injury induced by leukotoxin-stimulated bovine PMNs. In parallel studies, the protective effects of the same inhibitors on BPAEC injury induced by PMA-stimulated PMNs were also evaluated. Among these were SBTI, which inhibits trypsin and trypsin-like enzymes; a serine proteinase inhibitor which is also an elastase-specific inhibitor (CMK); an agent that inactivates H_2O_2 (catalase); DMTU, which reacts and scavenges both H_2O_2 and hydroxyl radical (HO·); a HO· scavenger (DMSO); a myeloperoxidase inhibitor (sodium azide); two 21-aminosteroid drugs (U74500A and U74389F) which are hydrophobic oxygen radical scavengers with iron-chelating ability (3, 5, 6, 17); and pentoxifylline, an agent that attenuates PMN activation and modulates its function (19, 35).

As the data in Table 1 indicate, SBTI at three different concentrations (1 to 100 μg/ml) provided less than 5%

TABLE 1. Effects of various inhibitors on BPAEC killing induced by *P. haemolytica* leukotoxin-stimulated bovine PMNs

Inhibitor	Concn	% Inhibition[a] (mean ± SD)	No. of expts[b]
SBTI	10 μg	3.2 ± 0.6	5
	100 μg	4.6 ± 1.1	5
CMK	7.5 μM	28.6 ± 2.5	3
	15 μM	43.6 ± 7.5	3
	30 μM	41.7 ± 6.6	3
Catalase	18 U	10.3 ± 1.8	5
	180 U	11.3 ± 3.9	5
	1,800 U	14.5 ± 2.1	5
DMTU	20 mM	31.4 ± 2.9	4
	40 mM	36.2 ± 1.86	4
	60 mM	40.32 ± 2.82	4
DMSO	10 μM	3.1 ± 0.89	3
	100 μM	12.8 ± 1.94	3
	200 μM	18.5 ± 3.93	3
Sodium azide	5 μM	1.7 ± 0.7	6
21-Aminosteroid 74389F	40 μM	0	4
	200 μM	2.6 ± 0.4	4

[a] Results are expressed as the percent inhibition (or protection) relative to that of the control that lacked inhibitor, by using the following formula: % inhibition = (% cytotoxicity of BPAEC-PMN coculture in the presence of leukotoxin − % cytotoxicity of BPAEC-PMN coculture in the presence of leukotoxin and inhibitor)/% cytotoxicity of BPAEC-PMN coculture in the presence of leukotoxin × 100. Calculation of the percent cytotoxicity was carried out as described in Materials and Methods.

[b] Each experiment had quadruplicate wells.

protection against BPAEC killing by either PMA- or leukotoxin-stimulated PMNs (five experiments). In contrast, coincubation with CMK at a concentration of 15 μM resulted in 43.6% ± 7.5% inhibition of BPAEC killing induced by leukotoxin-stimulated PMNs. CMK at the same concentration also inhibited significantly BPAEC killing induced by PMA-stimulated PMNs (48.3% ± 6.8%). Catalase (18 to 1,800 U/ml) inhibited BPAEC killing induced by both leukotoxin-stimulated (10.3% ± 1.8% to 14.5% ± 2.1% inhibition) and PMA-stimulated (11.0% ± 3.1% to 16.7% ± 3.7% inhibition) PMNs. As shown in Table 1, coincubation with DMTU at all three concentrations (20, 40, and 60 mM) employed significantly inhibited BPAEC killing induced by leukotoxin-stimulated PMNs (31 to 40% inhibition). DMTU at the same concentrations also inhibited BPAEC killing induced by PMA-stimulated PMNs (21 to 63% inhibition). In independent experiments, PMN-BPAEC cocultures were exposed to 20 mM DMTU, 15 μM CMK, or a mixture of 20 mM DMTU and 15 μM CMK and then stimulated with an optimal concentration of leukotoxin. Whereas DMTU and CMK alone provided 31.6% ± 6.4% and 39.2% ± 7.6% protection, respectively, the effect of both inhibitors was additive, providing 68.9% ± 9.7% protection against BPAEC killing (on the basis of three independent determinations). Additional experiments were done to investigate whether CMK or DMTU directly inhibited the ability of PMNs to mount a respiratory burst and lysosomal degranulation and altered their cytotoxicity after exposure to leukotoxin. Bovine PMNs were pretreated for 60 min at 37°C with three different concentrations of CMK (7.5 to 30 μM) or DMTU (20 to 60 mM), washed, and then exposed to an optimal concentration of leukotoxin. As described previously (25), generation of H_2O_2 was used as a measure of stimulation of respiratory burst, while release of arylsulfatase B was used to detect lysosomal degranulation, and cytotoxicity was assessed by measurement of release of lactate dehydrogenase. Results from three independent experiments showed that pretreatment of PMNs with various concentrations of CMK or DMTU and then exposed to leukotoxin produced nearly the same magnitude of respiratory burst, lysosomal degranulation, and cytotoxicity as seen with PMNs exposed to leukotoxin alone (data not shown).

DMSO (10 to 200 mM) also inhibited BPAEC killing induced by leukotoxin-stimulated PMNs (3.1% ± 0.89% to 18.5% ± 3.93%) or PMA-stimulated PMNs (6.0% ± 4.2% to 20.8% ± 3.2%). However, the myeloperoxidase inhibitor (sodium azide) was completely ineffective in protecting BPAEC from injury induced by leukotoxin- or PMA-stimulated PMNs (six experiments). As seen in Fig. 4 and 5, pretreatment of BPAEC with U74500A protected against BPAEC killing induced by leukotoxin- or PMA-stimulated PMNs in a dose-dependent fashion. Interestingly, U74500A at 200 μM significantly protected against BPAEC killing induced by both leukotoxin- and PMA-stimulated PMNs (100 and 86.9% protection, respectively). By contrast, pretreatment of PMNs or coincubation of PMN-BPAEC cocultures with this agent offered no protection against BPAEC injury induced by leukotoxin or PMA. Surprisingly, the other 21-aminosteroid, U74389F, at all concentrations employed (0.1 to 200 μM) was completely ineffective in protecting against BPAEC killing induced by either leukotoxin- or PMA-stimulated PMNs (four experiments). Finally, coincubation with pentoxifylline (5 and 10 mM) resulted in significant attenuation (44.2% ± 6.2% and 47.7% ± 7.3% protection, respectively) of BPAEC killing induced by PMA-stimulated PMNs but not leukotoxin-stimulated PMNs (Fig. 6). In contrast, pentoxifylline at 1 mM enhanced BPAEC killing induced by PMA-stimulated PMNs.

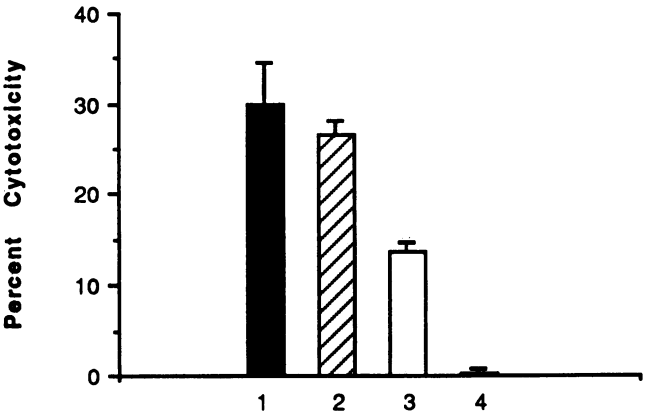

FIG. 4. Effect of U74500A on BPAEC killing induced by crude leukotoxin-stimulated PMNs. Confluent BPAEC monolayers in 48-well tissue culture plates were preincubated at 37°C with different concentrations of U74500A (10 μm [bar 2], 40 μm [bar 3], and 200 μm [bar 4]). Bar 1, no U74500A. After a 10-h incubation, the unincorporated drug was washed off and the BPAEC monolayers were labeled with ^{51}Cr as described in the text. PMNs were added to the BPAEC monolayers, and after a 30-min incubation, the cocultures were exposed to crude leukotoxin (40 U) and incubated for 18 h at 37°C. Data reflect the means ± SDs from quadruplicate samples in a single experiment that is representative of five.

DISCUSSION

The results of this study showed that the culture supernatant from *P. haemolytica* A1, referred to as crude leukotoxin, augmented PMN-mediated killing of BPAEC. However, in the absence of PMNs, there was minimal injury to the BPAEC. In accordance with results obtained from other laboratories (7, 29), we believe that the LPS component in the culture supernatant was responsible for the direct, albeit minimal, BPAEC injury in the absence of PMNs. Several lines of evidence demonstrated that the factor in the culture

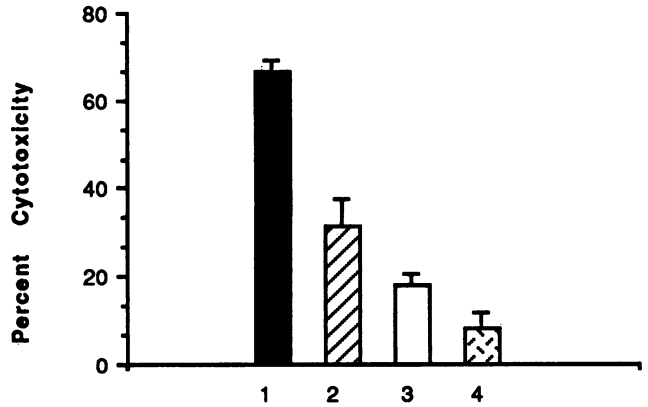

FIG. 5. Effect of U74500A on BPAEC killing induced by PMA-stimulated PMNs. The experiment was performed similarly to that described in the legend to Fig. 4, except that PMA (10 ng/ml) was used to stimulate the PMNs. Data reflect the means ± SDs from quadruplicate samples in a single experiment. The experiment was repeated five times with similar results.

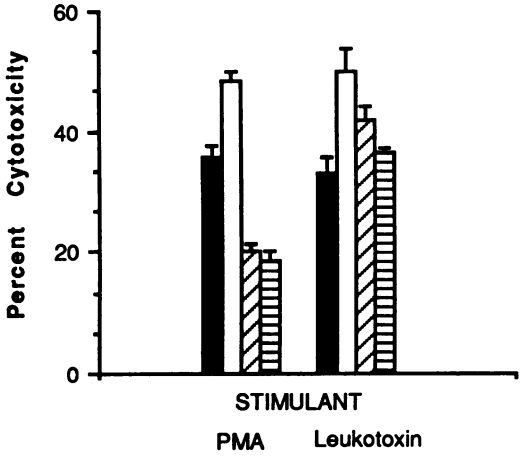

FIG. 6. Comparison of the effects of pentoxifylline on BPAEC killing induced by PMA or crude leukotoxin-stimulated PMNs. Various concentrations (1 mM [□], 5 mM [▨], and 10 mM [▤]) were mixed with PMNs and coincubated with ^{51}Cr-labeled BPAEC for 30 min ■, no pentoxifylline. The PMN-BPAEC cocultures were exposed to crude leukotoxin (40 U) or PMA (10 ng/ml) and incubated for 18 h at 37°C. Results are means ± SDs from quadruplicate samples in a single experiment that is representative of four.

supernatant responsible for augmenting the PMN-mediated killing of BPAEC was leukotoxin and not the contaminating LPS or capsular polysaccharide. First, we observed that heat-inactivated crude leukotoxin, which lost its leukocidal properties for PMNs but still contained capsular polysaccharide and retained endotoxic activity, failed to augment PMN-mediated killing of BPAEC. Secondly, coincubation with leukotoxin-neutralizing MAb-601 dose dependently blocked the ability of crude leukotoxin-stimulated PMN killing of BPAEC. Finally, polymyxin B treatment of crude leukotoxin, which neutralized endotoxic activity but retained leukotoxicity, failed to block PMN-mediated killing of BPAEC.

Previous studies from our laboratory (25) indicated that bovine PMNs stimulated by *P. haemolytica* leukotoxin generated a family of toxic oxygen radicals, including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), and also released various lysosomal proteases. This finding would implicate these toxic oxygen radicals and proteases from leukotoxin-stimulated PMNs in the damage to the pulmonary endothelium at sites of PMN attachment. Germane to the discussion on mechanisms of PMN-mediated endothelial cell injury, studies from several laboratories have shown that H_2O_2 generated extracellularly from activated PMNs readily penetrates the cell membrane, enters the endothelial cells, and reacts with intracellular iron to form highly reactive hydroxyl radical (HO·) via the Fenton reaction (13, 22, 40, 41). It is the HO· which is generated by the iron-catalyzed, H_2O_2-dependent pathway that is responsible for cytotoxicity of the endothelial cells by virtue of its ability to initiate lipid peroxidation and thereby damage cell membranes.

We designed experiments using various inhibitors and scavengers of H_2O_2 and HO· and proteinase inhibitors in our PMN-BPAEC coculture system to investigate the relative roles of these factors in BPAEC killing induced by leukotoxin-stimulated PMNs. Our observation that catalase offered a low degree of protection against BPAEC killing induced by leukotoxin-stimulated PMNs suggested a role for H_2O_2 in this killing. Perhaps the finding that DMTU provided a higher degree of protection than catalase against BPAEC killing merits an explanation. Studies by Kvietys et al. (22) suggest that H_2O_2 generated extracellularly from activated PMNs is relatively innocuous and is capable of causing only mild injury to endothelial cells. However, it can readily penetrate through the cell membrane of these cells and react with intracellular iron (Fenton reaction) to form a more destructive HO·. Because catalase (27, 36) is a large highly charged molecule which is impermeable, the current thought is that it can scavenge only extracellular H_2O_2. Thus, the low degree of protection we observed with catalase seems to be due to two reasons, first, H_2O_2 being a weak oxidant and, second, the limitation of catalase to scavenge only extracellular H_2O_2. By contrast, DMTU is a smaller molecule, is more membrane permeable, and can reach intracellular locations better than catalase and can scavenge H_2O_2 and HO· (14, 36). It is plausible, therefore, that the higher degree of protection provided by DMTU in our study (Table 1) may be attributed to its easy passage through the cell membrane of BPAEC and scavenging of both H_2O_2 and HO·. Our findings also demonstrate that DMSO, which is a known membrane-permeant HO· scavenger (27), significantly attenuated this BPAEC injury. However, the injury does not appear to involve myeloperoxidase products such as hypochlorous acid, as evidenced by the lack of protection afforded by sodium azide.

Our finding that the elastase-specific inhibitor CMK (Table 1) and not SBTI protected BPAEC from injury suggests that elastase released from degranulated lysosomes of leukotoxin-exposed PMNs also contributes to the injury. This was not surprising, because other investigators (18) have shown that CMK also inhibited neutrophil elastase-mediated injury of human umbilical vein endothelial cells. Furthermore, that the dual exposure of BPAEC to DMTU and CMK resulted in protection in an additive manner suggests that both elastase and HO· may be jointly contributing to BPAEC injury induced by leukotoxin-stimulated PMNs. This protective effect appears to be the result of inhibition of elastase activity and scavenging HO· rather than preventing the release of these substances from leukotoxin-exposed PMNs. Additional studies are needed, however, to clarify the mechanisms by which both substances additively protect against BPAEC injury.

The studies with the 21-aminosteroid U74500A are of particular interest. Pretreatment of the BPAEC with this substance (200 μm) afforded complete protection of BPAEC killing induced by leukotoxin-stimulated PMNs (Fig. 4). Also, U74500A at the same concentration significantly protected (86.9% ± 8.9% protection) against BPAEC injury induced by PMA-stimulated PMNs (Fig. 5). By contrast, pretreatment of the PMNs or coincubation of the PMN-BPAEC cocultures with this substance afforded no protection. In related studies, other investigators have documented intracellular iron in the endothelial cell as an important determinant in susceptibility to killing by activated PMNs. For example, Kvietys et al. (22) demonstrated that pretreatment of endothelial cells with deferoxamine, a potent iron chelator, markedly protected them from PMN-mediated injury. This supposition was strengthened by more recent studies by Balla et al. (3), who demonstrated that iron accumulated in endothelial cells greatly increased their susceptibility to oxidant damage and that U74500A completely prevented this injury. The precise mechanism of the protective effect of U74500A against BPAEC cytotoxicity caused by leukotoxin-stimulated PMNs is by no means clear. On the

basis of previous findings (3), this protective effect may be related to its iron-chelating property, thus preventing the generation of HO· by an iron-catalyzed, H_2O_2-dependent pathway. However, other described properties of U74500A, such as the ability to scavenge oxygen and lipid peroxyl radicals and inhibit iron-dependent lipid peroxidation of the endothelial cell membrane (5, 6), may also contribute to this protection. It was surprising that the other 21-aminosteroid, U74389F, afforded no protection against BPAEC killing induced by leukotoxin- or PMA-stimulated PMNs. U74389F is a close structural analog of U74006F (tirilazed mesylate) which is being clinically evaluated as an antioxidant to ameliorate lipid peroxidation-mediated acute and chronic neurodegenerative diseases in experimental animal models (4, 16). It is quite possible that despite this close similarity in their structure, U74389F differs from U74006F by being a weaker scavenger and inhibitor of iron-dependent lipid peroxidation.

It is interesting to find that pentoxifylline at higher concentrations (5 and 10 mM) significantly protected BPAEC from injury induced by PMA-stimulated PMNs (Fig. 6). In contrast, lower concentrations of pentoxifylline (1 mM) significantly enhanced BPAEC killing. Previous studies have shown that pentoxifylline enhanced the oxidative metabolism of PMNs at lower concentrations and suppressed the oxidative metabolism of PMNs at higher concentrations (19, 35). It has been suggested that this suppression of oxidative metabolism of PMNs by pentoxifylline is through an increase in intracellular concentrations of cyclic AMP (49). Thus, the attenuation of BPAEC killing of PMA-stimulated PMNs in the presence of high concentrations of pentoxifylline seen in our study may be the result of suppression of PMN oxidative metabolism. We cannot explain why pentoxifylline failed to protect BPAEC from injury induced by leukotoxin-stimulated PMNs. It is likely that leukotoxin by some as yet undefined mechanism inactivates the intracellular signal transduction pathways that lead to the elevation of cyclic AMP, thus interfering with the effects of pentoxifylline on neutrophils. Alternatively, perhaps the possibility that leukotoxin activation of PMNs is associated with intracellular signals other than cyclic AMP merits further study.

In summary, our data suggest that lung endothelial cell injury induced by leukotoxin-stimulated PMNs is mediated by the HO· (generated by an iron-catalyzed, H_2O_2-dependent pathway in the endothelial cell) and elastase released by the PMNs. Although these studies were performed using endothelial cells in culture, the finding that the 21-aminosteroid U74500A offered complete protection against this injury raises the possibility that this substance has therapeutic potential in pneumonic pasteurellosis. However, it should be reiterated that LPS from *P. haemolytica* also damages lung endothelial cells by both direct and indirect mechanisms (7, 28). We believe that this endothelial cell damage leads to edema and fibrin accumulation in the alveolar spaces seen in the disease. Continued research into the mechanisms involved in lung endothelial cell injury is crucial to control the disease.

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