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Pasteurella haemolytica Leukotoxin Enhances Production of Leukotriene B_4 and 5-Hydroxyeicosatetraenoic Acid by Bovine Polymorphonuclear Leukocytes

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The influence of the leukotoxin of *Pasteurella haemolytica* on the generation of arachidonic acid metabolites by bovine polymorphonuclear leukocytes (PMNs) was investigated. PMNs released 5-, 12-, and 15-hydroxyeicosatetraenoic acids (5-, 12-, and 15-HETE) and leukotriene B_4 (LTB_4) upon stimulation with arachidonic acid. The leukotoxin preparations dose dependently enhanced the release of the 5-lipoxygenase products 5-HETE and LTB_4 in arachidonic acid-stimulated PMNs, whereas the release of 12- and 15-HETE was not affected. The enhanced release of LTB_4 and 5-HETE was not due to a decreased cellular retention of the 5-lipoxygenase products. In addition, leukotoxin preparations by themselves were also able to induce LTB_4 and 5-HETE production in the absence of exogenous arachidonic acid. Generation of 5-lipoxygenase products by PMNs stimulated by leukotoxin may represent an important cellular event that occurs during infections with *P. haemolytica*.

Pasteurella species, especially *P. haemolytica* type A1, are major bovine respiratory pathogens. The bacterium has been associated with the advanced clinical signs, terminal lesions, and fatality of pneumonic pasteurellosis (shipping fever) of cattle (33). Morphologically, both naturally occurring and experimental infections result in pleuropneumonia with characteristic regions of coagulation necrosis demarcated by degenerate leukocytes (2). *P. haemolytica* produces a variety of potential virulence factors including an exotoxin (leukotoxin) (1). The live bacterium is toxic for bovine phagocytic cells such as alveolar macrophages and polymorphonuclear leukocytes (PMNs) in vitro (4, 28). This toxic effect is due to the secretion of the leukotoxin by actively growing bacteria (37). Biochemical studies have identified the toxin as a heat-labile, oxygen-stable, pH-stable, nondialyzable, water-soluble antigenic protein (11). The leukotoxin damages cells by forming transmembrane pores in the plasma membranes (13). Therefore, this leukotoxin is an important pathogenic factor in the early stage of *P. haemolytica*-induced pneumonia by diminishing phagocytic cell defense or, indirectly, by causing an inflammatory response mediated by constituents released by leukocytes exposed to leukotoxin (3).

PMNs are required for the acute lung injury induced by *P. haemolytica* infections (39). The leukotoxin alone is not toxic toward bronchial epithelium, but the severity of the lesions in the alveolar septa is dependent on the degree of leukocyte accumulation (46). Moreover, the *Pasteurella* bacterium by itself is able to produce a soluble substance that is directly chemotactic for bovine PMNs (8). In addition, other inflammatory mediators, including products released by leukocytes, can induce migration of PMNs. The 5-lipoxygenase products of arachidonic acid, leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE), are potent chemotactic agents (15, 17). These agents are known to induce recruitment of PMNs, thereby exacerbating inflammatory events and microvascular injury (29). Bovine peripheral blood PMNs and alveolar PMNs release LTB_4 and 5-HETE upon stimulation (20). Although in many studies the effects of *P. haemolytica* leukotoxin on the phagocytic and metabolic function of PMNs have been investigated, the influence of the leukotoxin on the metabolism of arachidonic acid has not been determined. Therefore, in our study we investigated the effects of *P. haemolytica* leukotoxin on arachidonic acid metabolism and found that the leukotoxin induced the release of 5-HETE and LTB_4 from bovine PMNs.

MATERIALS AND METHODS

***P. haemolytica* leukotoxin preparation.** *P. haemolytica* type A1 was grown in 100 ml of brain heart infusion at 37°C on a shaker. Leukotoxin was isolated by the method of Shewen and Wilkie (36). The bacteria were pelleted by centrifugation, suspended in 200 ml of RPMI 1640 medium (Flow Laboratories, Irvine, Scotland, United Kingdom) supplemented with 7% fetal calf serum, and incubated for 1 h at 37°C. After centrifugation, the supernatant fraction was filtered through a 0.45-μm-pore-size filter (Millipore, Molsheim, France), dialyzed against two changes of distilled water, lyophilized, and stored at 4°C. The same leukotoxin preparation was used in all experiments. Before use, the lyophilized material was reconstituted to the desired concentration in Krebs-bicarbonate buffer. The Krebs-bicarbonate buffer consisted of 118 mM NaCl, 4.7 mM KCl, 2.5 $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 8.3 mM glucose and was gassed with 95% O_2–5% CO_2 (pH 7.4). A concentration of 2 mg of leukotoxin per ml corresponded to the concentration before lyophilization. The viability of a PMN suspension (10^6 cells) was reduced by 5 and 20% as determined by trypan blue exclusion when the cells had been incubated with 63 and 125 μg of leukotoxin, respectively, for 30 min at 37°C in a total volume of 0.4 ml (21). The

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preparations contained less than 1% endotoxin as determined by the *Limulus* Amebocyte Lysate-5000 Automatic Endotoxin Detection System (Cape Cod Inc., Woods Hole, Mass.) at the Department of Medical Microbiology, University of Utrecht.

PMN suspensions. Peripheral blood samples were obtained from 6- to 15-week-old Friesian-Holstein calves by jugular vein puncture and collected in heparinized tubes. PMNs were isolated by lysis of contaminating erythrocytes and differential density centrifugation on Percoll (22). The cells were sedimented at 400 × *g* for 10 min and washed three times with Krebs-bicarbonate buffer. The final cell preparation contained 2×10^7 PMNs per ml with 85 to 90% neutrophils, 10 to 15% eosinophils, and less than 3% mononuclear cells. Viability was ≥95% as assessed by trypan blue exclusion. In some experiments, human PMNs were isolated from blood obtained from healthy volunteers by differential density centrifugation on Percoll and ammonium chloride lysis of contaminating erythrocytes.

Release of arachidonic acid metabolites. PMNs (2×10^7 cells per ml) were incubated with arachidonic acid (0 to 50 μM; Sigma Chemical Co., St. Louis, Mo.) and/or leukotoxin (0 to 0.5 mg/ml) in Krebs-bicarbonate buffer for 15 min at 37°C in a shaking water bath. The PMNs were subsequently sedimented at 750 × *g* for 15 min, and the supernatant fractions were applied to disposable reverse-phase extraction columns (J. T. Baker Chemicals, Deventer, The Netherlands), after which lipid-soluble material was eluted from the column with methanol (14).

In some experiments, PMNs were radiolabeled by incubation with 0.5 μCi of [^{14}C]arachidonic acid (56 Ci/mmol; NEN Du Pont de Nemours B.V., 's-Hertogenbosch, The Netherlands) per ml for 45 min at 37°C. The cells were subsequently washed three times and suspended in Krebs-bicarbonate buffer. More than 80% of the radiolabel was incorporated in the PMN preparation.

To determine the cellular and extracellular distribution of arachidonic acid metabolites, we collected supernatant fractions and cell pellets after incubation with leukotoxin. The cell pellets were resuspended in buffer, homogenized with a homogenizer (Ystral GmbH, Dottingen, Germany) at 17,500 rpm twice for 15 s each time, and further disrupted by three 20-s sonications. The disrupted cells and the supernatant fractions were extracted five times with 2 volumes of ether containing 0.5% acetic acid. The ether extracts were evaporated to dryness under vacuum, and the residues were dissolved in methanol for analysis.

Arachidonic acid-derived mediators were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) as described earlier (14). The analysis was performed at 30°C on a Nucleosil 5C18 column (250 by 4.6 mm; Chrompack, Middelburg, The Netherlands), and the elution was done with tetrahydrofuran-methanol-water-acetic acid (25:30:45:0.1; pH 5.5 with ammonium hydroxide) (47). The aqueous phase contained 0.1% EDTA to prevent binding to the column of cations. A flow rate of 0.9 ml/min was maintained, and the effluent was monitored at 270 and 235 nm, simultaneously. UV spectra were recorded by using a Pye Unicam PU 4021 multichannel photodiode array detector. Synthetic prostaglandin B_2 was used as an internal standard to correct for losses during the extraction procedures. The identification of the products was based on coelution with authentic standards on the RP-HPLC system and on UV absorption characteristics.

Statistical analysis. Grouped data are expressed as the mean ± standard error of the mean. Student's *t* test was applied for statistical evaluation of the results. *P* values exceeding 0.05 were considered not significant.

RESULTS

Several products were separated by RP-HPLC when extracted supernatant fractions from bovine PMNs incubated for 15 min with 50 μM arachidonic acid were analyzed (Fig. 1A). LTB_4 and 5-, 12-, and 15-HETE were the major metabolites identified. The amounts of arachidonic acid metabolites produced were influenced by adding leukotoxin to the incubation mixture (Fig. 1B). Twice as much LTB_4 and four times as much 5-HETE were released by the cells in the presence of leukotoxin compared with incubation of cells with arachidonic acid alone (Table 1). In contrast, leukotoxin did not influence the release of 12- and 15-HETE by arachidonic acid-stimulated bovine PMNs (Table 1). No effects on arachidonic acid metabolism were observed when heat-inactivated (30 min at 60°C) leukotoxin preparations or culture media were used (data not shown). Incubation of bovine PMNs with the calcium ionophore A23187 (10 μM) resulted in a production of 51 ± 6 ng of LTB_4 (n = three experiments). This level is comparable with the amount of LTB_4 produced by arachidonic acid-stimulated bovine PMNs in the presence of leukotoxin (Table 1), suggesting that this level represents the maximum LTB_4 generation of the bovine PMNs.

To investigate the species specifity of the leukotoxin (23), we did experiments to compare the effects of leukotoxin on the arachidonic acid metabolism of bovine and human PMNs. A marked increase in the release of LTB_4 (400%) and 5-HETE (300%) by arachidonic acid-stimulated bovine PMNs was observed in the presence of 0.5 mg of leukotoxin per ml compared with incubation of bovine PMNs with arachidonic acid alone. In contrast, leukotoxin did not influence the arachidonic acid metabolism of human PMNs (75% LTB_4 and 90% 5-HETE).

No arachidonic acid metabolites were detected with the RP-HPLC system when bovine PMNs were incubated with leukotoxin alone. However, to allow a more sensitive determination of the production of fatty acid metabolites by bovine PMNs, we labeled the cells with [^{14}C]arachidonic acid before the incubations. These radiolabeled PMNs (2×10^7 cells per ml) were incubated with or without 0.5 mg of leukotoxin per ml for 15 min at 37°C. Arachidonic acid metabolites were separated by RP-HPLC after extraction of the supernatant fluids, and the radioactivities of the eluates were counted in a liquid scintillation counter (n = three experiments). The results were as follows: in the absence of leukotoxin, LTB_4 release was 96 ± 85 dpm and 5-HETE release was 981 ± 244 dpm; in the presence of leukotoxin, LTB_4 release was 1,349 ± 208 dpm and 5-HETE release was 2,349 ± 571 dpm. It was therefore shown that leukotoxin preparations by themselves were able to increase the release of LTB_4 ($P < 0.05$) and 5-HETE ($0.05 < P < 0.1$) without exogenous arachidonic acid. No differences in the release of 12- and 15-HETE were observed between control incubations and leukotoxin-incubated cell suspensions (data not shown).

The increase in the quantities of LTB_4 and 5-HETE produced by arachidonic acid-stimulated bovine PMNs was dependent on the amount of leukotoxin present in the incubation mixture (Table 2). The release of LTB_4 and 5-HETE was already significantly potentiated with concentrations as low as 60 μg of leukotoxin per ml. A dose-dependent increase in the 5-lipoxygenase products was

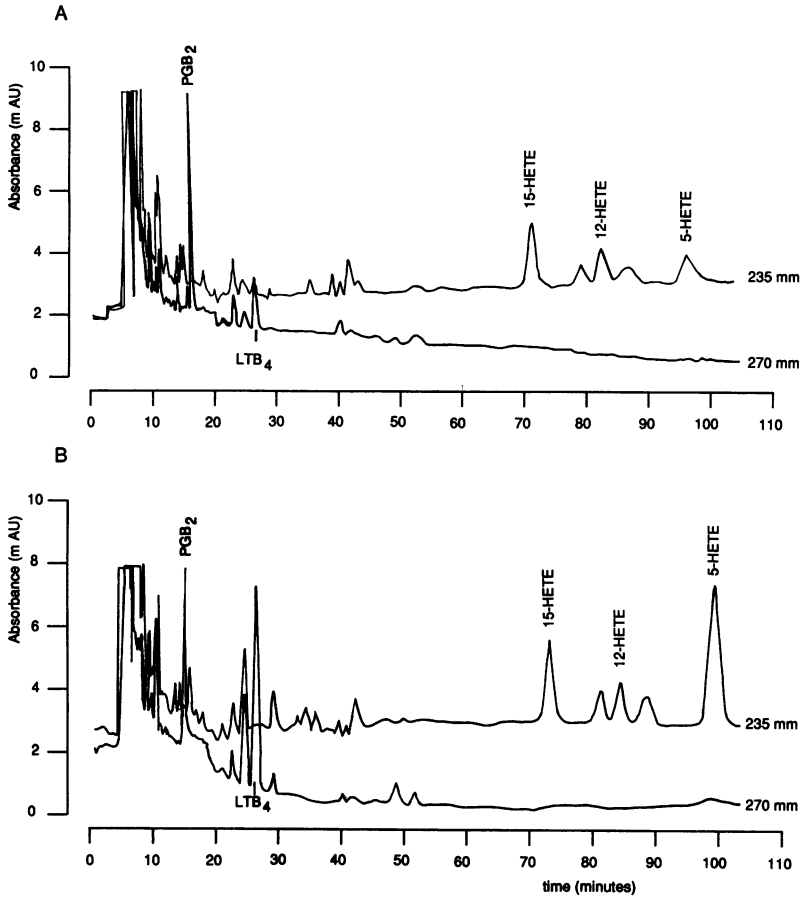

FIG. 1. RP-HPLC chromatograms of supernatant fluids of 2×10^7 PMNs incubated with 50 μM arachidonic acid in the absence (A) or presence (B) of 0.5 mg of leukotoxin per ml for 15 min at 37°C. Chromatograms shown are representative of 12 separate experiments. Synthetic prostaglandin B_2 (PGB_2) was used as an internal standard to correct for losses during the extraction procedures. AU, absorbance units.

measured between 0.03 and 0.13 mg of leukotoxin per ml (Table 2).

The intracellular and extracellular distribution of LTB_4 and 5-HETE generated by bovine PMNs was determined. Bovine PMNs were stimulated with arachidonic acid in the absence or presence of leukotoxin, and the amounts of arachidonic acid metabolites in the supernatant and cell fractions were measured. A total of 42% ± 2% of LTB_4 and 55% ± 9% of 5-HETE remained intracellular after incubation with 50 μM arachidonic acid alone. Only 21% ± 6% of LTB_4 and 40% ± 6% of 5-HETE remained intracellular in arachidonic acid-stimulated and leukotoxin-incubated bovine PMNs. However, the total amounts of intracellular and extracellular (i.e., released by the cells) LTB_4 and 5-HETE detected in the presence of leukotoxin were increased compared with incubation with arachidonic acid alone (174% ± 15% and 163% ± 16% increase, respectively; n = three experiments).

TABLE 1. Effect of leukotoxin on the release of arachidonic acid metabolites by PMNs[a]

Metabolite	Amt of metabolites (ng/ml) released	
	− leukotoxin	+ leukotoxin
LTB_4	23 ± 4	49 ± 5[b]
5-HETE	32 ± 9	120 ± 17[b]
12-HETE	66 ± 15	80 ± 18
15-HETE	54 ± 12	45 ± 9

[a] 2×10^7 cells were incubated with 50 μM arachidonic acid in the presence or absence of 0.5 mg of leukotoxin per ml for 15 min at 37°C. Release of arachidonic acid metabolites was measured by RP-HPLC after extraction of the supernatant fluids (n = 12 experiments).
[b] $P < 0.001$.

TABLE 2. Concentration-dependent effect of leukotoxin on the release of LTB_4 and 5-HETE by arachidonic acid-stimulated PMNs[a]

Leukotoxin (mg/ml)	LTB_4 (ng/ml)	5-HETE (ng/ml)	n
0	16 ± 3	15 ± 4	4
0.03	25 ± 6	45 ± 20	3
0.06	33 ± 5[b]	83 ± 21[b]	3
0.13	43 ± 4[c]	129 ± 33[b]	4
0.25	38 ± 3[c]	117 ± 36[b]	3
0.50	37 ± 4[c]	124 ± 20[c]	4

[a] 2×10^7 cells were incubated with 50 μM arachidonic acid in the presence of 0 to 0.5 mg of leukotoxin per ml for 15 min at 37°C. Release of arachidonic acid metabolites was measured by RP-HPLC after extraction of the supernatant fluids.
[b] $P < 0.05$.
[c] $P < 0.01$.

DISCUSSION

P. haemolytica is the principal microorganism associated with bovine pneumonic pasteurellosis, and its leukotoxin has been implicated as a virulence factor in that disease (3, 33, 37). The leukotoxin is able to decrease the phagocytic and metabolic cell functions of PMNs in vitro (4, 10, 21). This present study investigated whether the leukotoxin of *P. haemolytica* affected the production of arachidonic acid metabolites of bovine PMNs. It was found that bovine PMNs released LTB_4 and 5-, 12-, and 15-HETE upon incubation with arachidonic acid. The leukotoxin preparation potentiated the release of only the 5-lipoxygenase products 5-HETE and LTB_4, whereas the release of 12- and 15-HETE was not influenced.

Arachidonic acid is not only a substrate but also activates lipoxygenases (25, 31, 38). Calcium is one of the factors required for the activation of the 5-lipoxygenase, whereas the 12- and 15-lipoxygenases can be activated in the absence of calcium (30). Indeed, stimulation of PMNs with the calcium ionophore A23187 results predominantly in the production of 5-HETE and LTB_4 without detectable levels of 12- and 15-lipoxygenase products (20, 41). It has been shown that bovine PMNs are capable of synthesizing 5-HETE, 12-HETE, and LTB_4 (20, 48). However, the production of 12-HETE by the cell preparations in our experiments may also represent platelet contamination in the suspensions because 12-HETE is the major lipoxygenase product of platelets (25, 26). Production of 5-, 12-, and 15-lipoxygenase products by bovine alveolar macrophages can be achieved by a combined challenge of the cells with arachidonic acid and the calcium ionophore A23187 (44). The 15-lipoxygenase pathway is the predominant pathway for arachidonic acid in human eosinophils (38, 45), and the detection of 15-HETE in our experiments may represent 15-lipoxygenase activity of bovine eosinophils present in the cell suspensions. The ability of arachidonic acid to activate the lipoxygenases in our cell preparations may be of pathophysiological importance in vivo because the concentrations of arachidonic acid required to activate PMNs can be achieved in inflammatory lesions (18). Moreover, in the absence of exogenous arachidonic acid, the leukotoxin was able to induce the release of LTB_4 and 5-HETE as indicated by the experiments with radiolabeled PMNs.

A genetic relationship exists between the *P. haemolytica* leukotoxin and the hemolysin of *Escherichia coli* (27, 40). The *E. coli* hemolysin has a wide range of target cells from different species (9, 16, 24), whereas the *P. haemolytica* leukotoxin only attacks ruminant leukocytes (23, 36). These bacterial cytolysins generate transmembrane pores in the susceptible cells, resulting in a release of granule constituents from leukocytes and a decrease of cell functions (5, 6, 12, 13). Some of these and other pore-forming cytolysins have been shown to affect the arachidonic acid metabolism of leukocytes and tissues. The *E. coli* hemolysin is able to induce the generation of leukotrienes and HETEs in blood-free perfused lungs (19) and human PMNs (34). The arachidonic acid pathway in pulmonary endothelial cells and PMNs is stimulated by *Staphylococcus aureus* alpha-toxin (42, 43). *S. aureus* delta-toxin modulates the generation of LTB_4 induced by calcium ionophore (increase) and opsonized zymosan (decrease) in human PMNs (32). Incubation of human PMNs with streptolysin O leads to the generation of leukotrienes (7).

Several possible mechanisms by which the *P. haemolytica* leukotoxin exerts its effects on arachidonic acid metabolism can be considered. A fraction of the arachidonic acid metabolites could remain intracellular rather than being released by PMNs (49). The leukotoxin induces pores and subsequently membrane damage and lysis of the cells. It is possible that the enhanced amount of 5-lipoxygenase products measured was due to an increase in the release of 5-HETE and LTB_4 without extra intracellular production. If this assumption is true, less LTB_4 and 5-HETE would be present in the cell fractions after the incubation procedure with leukotoxin compared with control incubations. However, the total amounts of LTB_4 and 5-HETE present in the cell fraction after incubation with leukotoxin were also increased in arachidonic acid-stimulated PMNs compared with incubations with only arachidonic acid. From biochemical and pharmacological studies with *S. aureus* alpha-toxin and *Pseudomonas aeruginosa* cytotoxin, there is evidence that the sequence of events includes transmembrane pore formation, toxin-related calcium gating, calcium-mediated induction of phospholipase activities, and subsequent formation of cell-specific arachidonic acid metabolites (35, 43). An influx of calcium due to the transmembrane pores may trigger lipid mediator generation by activating the phospholipase A_2 and/or the 5-lipoxygenase enzyme systems. *P. haemolytica* leukotoxin acts rapidly on cultured bovine lymphoma cells to cause release of intracellular potassium and uptake of extracellular calcium (12, 13). The hypothesis that the increased 5-lipoxygenase product formation is due to a calcium influx caused by transmembrane pores induced by the leukotoxin is supported by the observation that only the amount of 5-lipoxygenase products increased while the amounts of 12- and 15-HETE did not differ between control and leukotoxin-incubated PMNs.

LTB_4 and 5-HETE are important mediators in inflammatory reactions because they are chemotactic agents for granulocytes (15, 17). Therefore, they may serve as biological amplifiers in the inflammatory process by inducing a further accumulation of PMNs at the site of injury (29). The capacity of the leukotoxin to induce and enhance the generation of LTB_4 and 5-HETE could be relevant for amplification of the inflammatory reactions and the damage observed in the lungs during infection with *P. haemolytica*.

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