INFECTION AND IMMUNITY, Sept. 1997, p. 3719–3724 Vol. 65, No. 9
0019-9567/97/$04.00+0

Binding of *Pasteurella haemolytica* Leukotoxin to Bovine Leukocytes

J. F. BROWN, F. LEITE, AND C. J. CZUPRYNSKI*

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin 53706

Received 18 February 1997/Returned for modification 24 March 1997/Accepted 25 June 1997

Pasteurella haemolytica **is the principal bacterial pathogen in the bovine respiratory disease complex. This organism produces an exotoxin (referred to as leukotoxin) during logarithmic-phase growth that is a potent leukocyte-modulating agent. At low concentrations, it activates neutrophils and mononuclear phagocytes to release inflammatory mediators, while at the same time making these cells destined to undergo apoptotic cell death. At higher concentrations, the toxin causes rapid swelling and loss of cell viability. In this study, we demonstrated that toxin binding can be directly evaluated by flow cytometry with biologically active biotinylated leukotoxin. Leukotoxin binding was blocked by the addition of a neutralizing anti-leukotoxin monoclonal antibody and was not detected when bovine leukocytes were incubated with culture filtrates from a mutant strain of *P. haemolytica* that does not produce biologically active leukotoxin. In addition, treatment of bovine leukocytes with protease K eliminated subsequent binding of leukotoxin, suggesting that there is a protein on the leukocyte surface that is either a leukotoxin binding site or is required for stabilization of leukotoxin binding. We did not detect binding of biotinylated leukotoxin to porcine or human leukocytes, which have been reported previously to be resistant to the lytic effects of the leukotoxin. These findings suggest that there may be a specific binding site for *P. haemolytica* leukotoxin on bovine but not on porcine or human leukocytes and that it might be involved in the activation and lytic activities of the leukotoxin.**

Pasteurellosis is a major economic problem for cattle and sheep industries throughout the world. Bovine pasteurellosis, which is caused by infection of the lung with *Pasteurella haemolytica* A1, is estimated to cause more than $800 million in annual losses for the U.S. beef cattle industry (16). *P. haemolytica* A1 normally resides in the tonsillar crypts as part of the microbial flora of healthy cattle (16). However, when cattle are stressed by shipment, or experience active viral infection, *P. haemolytica* A1 increases in number and is inhaled into the lung, where it multiplies rapidly (10). During logarithmic growth, it secretes a 104-kDa leukotoxin (LKT) that has a wide array of biological effects on bovine leukocytes (1, 40, 41). At low concentrations, the LKT stimulates bovine neutrophils and mononuclear phagocytes to produce reactive oxygen intermediates, to degranulate, and to release eicosanoids and cytokines (8, 13, 20, 25, 26, 33, 38, 43, 45, 48). *P. haemolytica* also stimulates bovine alveolar macrophages to express procoagulant activity on their surface, which can amplify fibrin deposition (6, 35). Presumably this activity, which can even be expressed on dead alveolar macrophages, is caused at least in part by the LKT. LKT inhibits bovine lymphocyte proliferation, and major histocompatibility complex class II antigen expression, without necessarily causing cell death (12, 21, 27). Similar concentrations of LKT cause bovine leukocytes to undergo apoptosis (44), whereas greater concentrations of LKT cause rapid swelling and lysis of leukocytes (1, 9, 11, 33, 41, 45). The net result of these activities is severe inflammation of the lung, as exemplified by the fibrinous pleuropneumonia of acute pulmonary pasteurellosis in cattle (i.e., "shipping fever") (10, 42).

The *P. haemolytica* LKT is a member of a large family of exotoxins produced by gram-negative bacteria. Known as the RTX family (for repeat in toxin), these toxins share a similar synthesis and secretion system and exhibit related biological activities (30, 47). The N-terminal ends of RTX toxins possess several lipophilic regions that are thought to cause pore formation, or other membrane defects, in susceptible cells (3, 4, 23, 32). These cells then exhibit increased uptake of extracellular Ca^{2+}, loss of normal membrane permeability, and release of K^+ and macromolecules such as ATP (9, 18, 45, 46).

The mechanism by which the LKT causes these changes in bovine leukocytes has not been delineated. The specificity of the LKT for ruminant leukocytes, and the lack of an effect on leukocytes from other species (41), suggests there is a specific binding site on ruminant leukocytes. However, such an LKT binding site (i.e., receptor) has not been previously described. Efforts have been made to identify binding of the related *Escherichia coli* hemolysin, and other RTX toxins, to leukocytes and erythrocytes (2, 5, 11, 15, 22, 24, 36, 37, 39). However, these have usually relied on cell death as an indirect indication of toxin binding rather than direct measurement of the binding event itself.

The hypothesis addressed in this study was that binding of *P. haemolytica* LKT to leukocytes can be demonstrated by flow cytometry and that binding reflects the biological specificity of LKT for leukocytes. We succeeded in demonstrating binding of biotinylated LKT to bovine leukocytes. Binding was not detected when we used biotinylated culture filtrates from an LKT mutant of *P. haemolytica*. Binding of active LKT was blocked by the addition of a neutralizing anti-LKT monoclonal antibody (MAb) and was dependent on the presence of a protease K-sensitive molecule on the leukocyte surface. Furthermore, LKT did not bind to mammalian species of leukocytes (i.e., pig and human) for which it has no biological activity. These findings suggest there is a relationship between the binding of *P. haemolytica* LKT and its biological effects on leukocytes.

* Corresponding author. Mailing address: Department of Pathobiological Sciences, 2015 Linden Drive West, Madison, WI 53706. Phone: (608) 262-8102. Fax: (608) 263-0438. E-mail: czuprync@svm.vetmed.wisc.edu.

MATERIALS AND METHODS

Leukocyte preparation. Peripheral blood was collected from healthy Holstein donor cows with vacutainer tubes (Becton-Dickinson, Rutherford, N.J.) containing sodium citrate (0.38% final volume) as anticoagulant. The blood was centrifuged (250 × *g* for 20 min), and the platelet-rich plasma was removed. The blood cells were centrifuged again (650 × *g* for 20 min), and the buffy coat cells were collected. Peripheral blood mononuclear cells (PBMCs) were obtained as described previously (43). Briefly, the buffy coat cells were diluted 1:4 (vol/vol) in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS) with 0.25% bovine serum albumin (BSA) and 10 mM EDTA (HEDTA) and overlaid on Nycoprep gradients (1.081 density; Nycomed, Oslo, Norway). These were centrifuged (350 × *g*) at room temperature for 30 min, and the mononuclear cell fractions were collected, washed four times at 4°C in Ca^{2+}- and Mg^{2+}-containing HBSS, and resuspended in RPMI 1640 with 5% fetal bovine serum at 10^7 cells/ml. Neutrophils were obtained as described previously (43) by rapid hypotonic lysis and centrifugation through a Percoll gradient (Pharmacia, Uppsala, Sweden). The neutrophil pellets were washed twice in HBSS and resuspended at 10^7 cells/ml in HBSS, or in RPMI 1640, with 5% fetal bovine serum. These cell suspensions were greater than 95% neutrophils, as determined by evaluation of Diff-Quik-stained cytocentrifuge smears. Both neutrophil and mononuclear cell populations were greater than 95% viable, as estimated by trypan blue exclusion.

***Pasteurella haemolytica*.** Two strains of *P. haemolytica* A1, obtained from G. Weinstock (Houston, Tex.), were used in this study. The first of these (strain 59B049) was a wild-type strain isolated from a pneumonic bovine lung. This parent strain was mutagenized with nitrosoguanidine to obtain a leukotoxin mutant (strain 59B0071) that lacked detectable leukotoxin activity but was unaltered in rate of growth, antibiotic susceptibility, and production of other known virulence determinants (7). Consistent with its lack of leukotoxin production, mutant strain 59B0071 exhibited reduced virulence for experimentally infected calves (34). Although we observed a protein with a molecular mass (estimated as 100 kDa) similar to that of biologically active LKT in the culture filtrates of this *P. haemolytica* mutant, it has been reported that this mutant does not produce LKT, as detected by enzyme-linked immunosorbent assay or Western blot analysis (34), and we consistently detected no leukotoxic activity or leukocyte binding by its culture filtrates.

Leukotoxin production and purification. All leukotoxin preparations were produced and partially purified as described previously (13, 44). Briefly, *P. haemolytica* A1 was inoculated onto blood agar and incubated overnight (Remel, Lenexa, Kans.) at 37°C. The bacteria were washed from the agar surface with 10 ml of brain heart infusion broth that contained 0.5% yeast extract (BHI/YE; Difco, Detroit, Mich.) and incubated at 37°C for 1 h while rotating (8 rpm) in 15-ml polypropylene tubes. For each bacterial strain, a 10-ml aliquot of this suspension was then used to inoculate 200 ml of BHI/YE in a 500-ml Erlenmeyer flask. The flask was then incubated for 2 h at 37°C with gentle shaking. The bacteria were then collected by centrifugation (1,600 × *g* for 15 min), resuspended in 200 ml of RPMI 1640 supplemented with L-glutamine (4.0 mM), and incubated on a shaker apparatus for 4 h at 37°C. The bacteria were harvested by centrifugation (1,600 × *g* for 20 min), and the crude leukotoxin-containing supernatant was collected and passed through a 0.45-mm-pore-size bottletop filter (Nalgene, Rochester, N.Y.) to remove any residual bacteria. Aliquots (20 ml) of crude leukotoxin were concentrated over an Amicon ultrafiltration unit equipped with a 62-mm-diameter XM-50 ultrafiltration membrane. The volume was then reduced to 10 to 20 ml over a 1- to 2-h period by applying 60-psi transmembrane pressure with nitrogen gas. The partially purified leukotoxin preparation that remained was then collected and stored as 5-ml aliquots at −70°C. To further purify the leukotoxin, aliquots were thawed, concentrated to approximately 0.5 ml, and chromatographed by size-exclusion high performance liquid chromatography (HPLC) with a GPC-500 (or in some instances GPC-100) sizing column (Alltech). To do this, the LKT (0.5 ml) was loaded onto a column (1 by 25 cm) at a flow rate of 2 ml/min with an elution buffer of 50 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM dithiothreitol (pH 7.4). Leukotoxic activity, which eluted in approximately 7 to 8 min, was confirmed by trypan blue exclusion in bovine peripheral blood leukocytes incubated with eluted fractions for 30 min at 37°C. When we assessed the leukotoxic activity (where 1 U is defined as the dilution causing 50% killing of bovine peripheral blood leukocytes as determined by trypan blue exclusion), we found that the crude leukotoxin had 881 ± 1.5 U of activity per mg of protein, the ultrafiltration-concentrated leukotoxin had 5,805 U per mg of protein, and the HPLC-chromatographed leukotoxin had 12,170 ± 630 U per mg of protein. To verify the purification of the LKT, an approximately 2-μg sample was loaded onto a Vydac C-18 microbore reversed-phase HPLC column and eluted with a linear gradient of trifluoroacetate (TFA) and acetonitrile (buffer A, 0.1% TFA in H_2O; buffer B, acetonitrile-TFA). This yielded a sharp peak of protein, suggesting that the LKT preparation was relatively pure (data not shown). Purified LKT was stored at −80°C until used in an experiment.

Biotinylation of LKT. To biotinylate the LKT, an 80:1 molar ratio of NHS-LC-biotin (Pierce Chemical, Rockford, Ill.) to purified LKT was incubated in an ice bath for 20 min. The mixture was then concentrated to 1 ml in a prechilled Amicon Centricon tube (50-kDa cutoff), and the reaction was stopped by the addition of crystalline BSA (30 mg) and incubated at 4°C for 30 min. Unbound biotin was eliminated by buffer exchange over a Sephadex G-25 column (1 by 25 cm) with phosphate-buffered saline (PBS) (pH 7.2) as elution buffer. The LKT eluted in the void volume in 7 to 8 min, as monitored by absorbance at 280 nm. Total protein in the eluted LKT (prior to the addition of BSA) was determined by using the microplate Bradford assay (43). Leukotoxicity was assessed by incubating biotinylated LKT with bovine peripheral blood leukocytes for 30 min at 37°C, followed by trypan blue exclusion. We routinely found that biotinylation did not substantially diminish leukotoxic activity (12,170 ± 630 U per mg of protein and 12,258 ± 1,808 U per mg of protein before and after biotinylation, respectively). If a preparation contained substantially reduced leukotoxic activity, it was discarded.

Protease K treatment. Suspensions of PBMCs or peripheral blood neutrophils (PMNs) (5×10^6 cells in a 0.2-ml volume) were incubated with 100 μg of protease K (Sigma) for 5 min at room temperature. The cells were then washed and resuspended in HBSS. If cell clumping occurred, the cell suspension was filtered through a nylon mesh (pore size, 40 μm). Cell viability was not adversely affected by protease K treatment, as assessed by trypan blue exclusion. In five separate experiments, the mean ± standard error of the mean (SEM) percentages of viable cells for protease K-treated and control PMNs were 95.8 ± 2.4 and 97.2 ± 1.0, respectively, and for protease K-treated and control PBMCs they were 98 ± 0.9 and 90.3 ± 2.9, respectively.

To estimate the possible carryover of protease K on treated cells, we determined the proteolytic activity of protease K-treated and control cells by using a casein hydrolysis assay. Cell suspensions, or the supernatant of cells washed with HBSS, were added to a tube containing 0.2 ml of casein solution (2 mg per ml in PBS [pH 7.2]) and incubated at 37°C for 45 min. Controls included tubes with a known amount of protease K (4 μg) or casein alone. The tubes were then cooled in an ice bucket, and the intact casein was precipitated with 5% (vol/vol) trifluoracetic acid. The tubes were centrifuged at 14,000 × *g* for 10 min, and the supernatant was removed to estimate degraded peptide by using the bicinchoninic acid colorimetric assay (Pierce Chemical).

Flow cytometry. Bovine PMNs or PBMCs were incubated with biotinylated LKT (10 to 20 μg of LKT per 2×10^6 to 5×10^6 cells) for 45 min at 4°C. In later experiments we learned that LKT binding could be detected with as little as 1.5 μg of LKT per 5×10^6 cells and that incubation for as little as 10 min at 4°C gave maximal binding (data not shown). Cells were washed once and resuspended in HBSS. Extra-avidin–fluorescein isothiocyanate (FITC) (4 μl) (Sigma) was added, and the cells were incubated for 30 min at 4°C. The cells were washed once with 15 ml of HBSS and resuspended in 1 ml of HBSS. The stained cells were fixed with 0.4% paraformaldehyde (final concentration) and analyzed by flow cytometry with a Coulter Epics-C flow cytometer (5,000 to 50,000 cells were scored for green fluorescence on a log scale). Controls included cells incubated in medium alone, or incubated with biotinylated culture filtrate from the *P. haemolytica* LKT mutant, before exposure to extra-avidin–FITC.

LKT cross-linking. Approximately 5×10^7 PMNs or PBMCs were suspended in 5 ml of PBS. Biotinylated toxin was added (100 μg), and the cells were incubated at 4°C for 45 min. The cross-linking agent DSS (Pierce Chemical) was dissolved in dimethyl sulfoxide (1 mg/ml), added to the toxin-labelled cells at a final concentration of 300 μg/ml, and held in the refrigerator for 18 h. The mixture was then centrifuged, and the cell pellet was washed with PBS. The washed pellet was resuspended in 200 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol) and boiled for 5 min. The total protein in the cell pellets was estimated to be approximately 10 mg as determined by the Bradford assay. The samples were loaded at 5, 15, and 30 μl in replicate lanes of a 10% minigel and cast with a 5.5% stacking gel. A mixture of biotinylated molecular weight standards (Sigma) was included on each of two gels. After completion of the run, the gels were blotted onto nitrocellulose membranes (Amersham) for 3 h at 500 mA (approximately 50 V). The blots were blocked overnight in 2% BSA in PBS, washed briefly with PBS, and probed with extra-avidin–alkaline phosphatase (Sigma) for 45 min at room temperature (1:2,000 dilution). After 3 washes with PBS, the blots were developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium substrate (Sigma).

Leukotoxin-neutralizing MAb. A murine MAb (immunoglobulin G1) that neutralizes the cytolytic effects of *P. haemolytica* LKT was generously provided by S. Srikumaran (Lincoln, Nebr.). Preparation (13) and use of this MAb (MM601) (7, 33, 43, 44) have been described previously.

Statistical analysis. Data were analyzed for statistical significance using a repeated measures analysis of variance, followed by the Tukey-Kramer multiple comparisons test as performed by the Instat software program (GraphPad, San Diego, Calif.).

RESULTS

Detection of biotinylated LKT binding to bovine leukocytes by flow cytometry. After determining that the biotinylated LKT retained its cytotoxic activity (as assessed by uptake of trypan blue by LKT-treated bovine peripheral blood leukocytes), it was used as a probe to detect LKT binding to bovine leukocytes by flow cytometry. Purified bovine PMNs and PBMCs were separately incubated with biotinylated LKT for approxi-

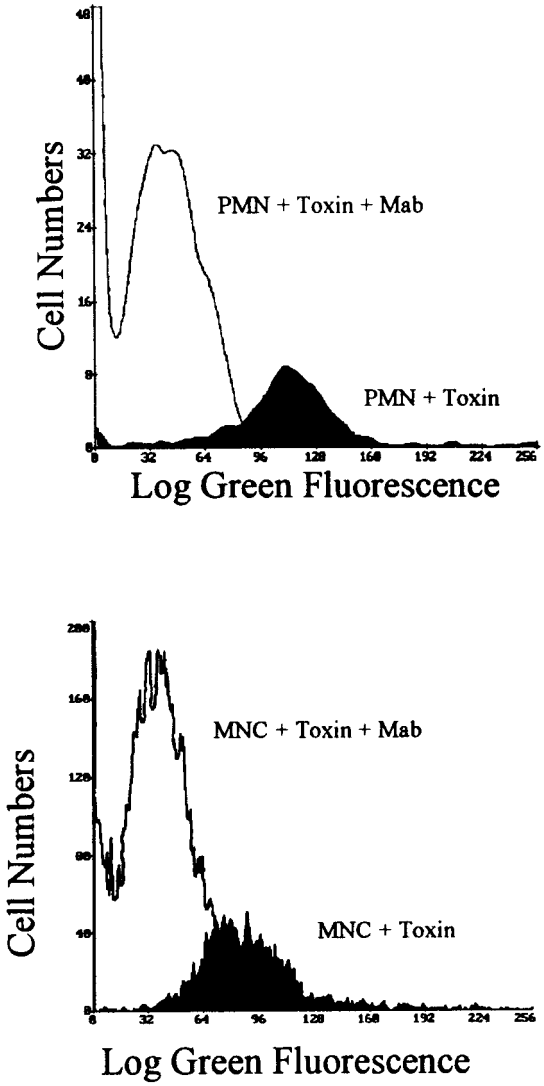

FIG. 1. Representative histograms illustrating the binding of biotinylated LKT to bovine PMNs and PBMCs (MNC) as detected by the addition of extra-avidin–FITC. Staining was reduced to baseline by the addition of a neutralizing anti-LKT MAb (MM601). Results are from a single representative experiment.

mately 45 min at 4°C as described in Materials and Methods. The cells were then incubated with extra-avidin–FITC and analyzed by flow cytometry using a Coulter Epics C flow cytometer. As shown in Fig. 1, we consistently observed a sharp peak of LKT binding to bovine PMNs. Incubation of PMNs with leukotoxin in the presence of a neutralizing anti-LKT MAb (MM601) eliminated LKT binding (Fig. 1 and Table 1), whereas incubation with a nonneutralizing anti-LKT antibody, or control MAbs, did not prevent binding (data not shown). Binding appeared to require biologically active leukotoxin. Heating LKT at 60 or 70°C for 30 to 60 min (which results in a substantial drop in leukotoxic activity) reduced maximal binding, as detectable by flow cytometry, by 73% (mean of two experiments). Furthermore, preincubation of cells with heat-activated LKT did not reduce subsequent binding of biologically active biotinylated LKT as detected by flow cytometry (data not shown). A lower percentage of PBMCs than PMNs bound LKT (Fig. 1), and binding of LKT to PBMCs was blocked by addition of the neutralizing anti-LKT MAb (Fig. 1 and Table 2). We also assessed the ability of culture filtrates from a mutant strain of *P. haemolytica* that does not produce biologically active LKT (7) to bind to bovine PMNs and PBMCs. We did not detect significant binding of this mutant LKT preparation to either bovine PMNs (Table 1) or PBMCs (Table 2).

TABLE 1. Flow cytometric analysis of LKT binding to bovine PMNs

LKT	Protease K treatment[a]	Anti-LKT MAb[b]	Percent positive cells[c]
−	−	−	7.7 ± 2.9
+	−	−	63.9 ± 20.0[d]
+	+	−	4.0 ± 2.7
Mutant[e]	−	−	5.6 ± 3.4
+	−	+	6.6 ± 2.7

[a] PMNs were pretreated with protease K and washed before incubation with biotinylated LKT.
[b] Biotinylated LKT was preincubated with anti-LKT MAb MM601 before being added to cells.
[c] Data are means ± SEM of three separate experiments.
[d] $P < 0.01$ versus all other groups as analyzed by repeated measures analysis of variance and the Tukey-Kramer multiple comparisons test.
[e] Incubation of PMNs with biotinylated culture filtrate from an LKT mutant strain of *P. haemolytica.*

Effects of protease K treatment on LKT binding to bovine leukocytes. We also examined the possibility that there may be a surface protein associated with LKT binding to bovine leukocytes. To do this, we pretreated leukocytes with protease K, under conditions that did not result in the loss of leukocyte viability, and then incubated the leukocytes with biotinylated LKT. Our results indicated that treatment with protease K dramatically reduced binding of LKT to bovine PMNs and PBMCs compared with control leukocytes (Tables 1 and 2). Extensive washing of the protease K-treated cells did not alter their susceptibility to killing by LKT (data not shown), suggesting that the absence of binding did not reflect the effect of residual protease K inactivating LKT in the reaction tubes.

***P. haemolytica* LKT does not bind porcine leukocytes.** It has been reported previously that the lethal activity of the *P. haemolytica* LKT is restricted to bovine and other ruminant species of leukocytes and that cells from other mammalian species tested are unaffected (41). We tested whether LKT binding might occur in the absence of biological activity by incubating porcine PMNs and PBMCs with biotinylated LKT in vitro. We observed little or no binding of LKT to these cells (Table 3),

TABLE 2. Flow cytometric analysis of LKT binding to bovine PBMCs

LKT	Protease K treatment[a]	MM601 MAb[b]	Percent positive cells[c]
−	−	−	2.8 ± 1.7
+	−	−	9.1 ± 0.9[d]
+	+	−	2.4 ± 1.0
Mutant[e]	−	−	2.4 ± 0.4
+	−	+	3.3 ± 1.3

[a] PBMCs were pretreated with protease K and washed before incubation with biotinylated LKT.
[b] Biotinylated LKT was preincubated with anti-LKT MAb MM601 before being added to cells.
[c] Data are means ± SEM of three separate experiments.
[d] $P < 0.01$ versus all other groups as analyzed by repeated measures analysis of variance and the Tukey-Kramer multiple comparisons test.
[e] Incubation of PBMCs with biotinylated culture filtrate from an LKT mutant strain of *P. haemolytica.*

TABLE 3. Flow cytometric analysis of LKT binding to porcine and bovine PMNs and PBMCs

Species	LKT	MM601 MAb	Percent positive cells[a]	
			PMNs	PBMCs
Porcine	+	−	3.2 ± 0.9	3.5 ± 0.9
	+	+	3.5 ± 0.9	5.7 ± 2.0
	Mutant[b]		4.2 ± 1.5	5.0 ± 1.8
	−	−	3.5 ± 1.8	5.3 ± 0.6
Bovine	+	−	40.9 ± 3.3	35.5 ± 12.4
	+	+	6.2 ± 1.3	4.2 ± 1.0
	Mutant[b]	−	5.6 ± 0.5	4.8 ± 0.2

[a] Results were obtained with six separate porcine and three separate bovine donors. Data are means ± SEM.

[b] Incubation of PMNs and PBMCs with biotinylated culture filtrate from an LKT mutant strain of *P. haemolytica.*

suggesting that the inability of *P. haemolytica* LKT to kill this species' leukocytes may be related to its inability to bind to the leukocyte surface. In two experiments, we also did not detect binding of LKT to human leukocytes (data not shown).

DISCUSSION

This study describes a new flow cytometry assay for detecting the binding of *P. haemolytica* LKT to bovine leukocytes. Our results suggest that biologically active LKT will bind to bovine PMNs and PBMCs, with the former demonstrating a stronger signal than the latter. Reasons for this are not clear, but prior publications suggest that neutrophils are particularly susceptible to both the activating and lytic effects of the *P. haemolytica* LKT and other RTX toxins (1, 4, 8, 13, 14, 19, 20, 23, 25, 26, 33, 38, 42–45). Lack of detectable binding by biotinylated culture filtrates from a *P. haemolytica* LKT mutant suggests that binding was dependent on the presence of active LKT and was not due to other *P. haemolytica* components (such as lipopolysaccharide) in the culture filtrate. Furthermore, binding of biologically active LKT to bovine PMNs and PBMCs was blocked by addition of the neutralizing anti-LKT MAb MM601 (17). It is interesting that we did not detect binding when biotinylated LKT was incubated with porcine or human leukocytes, which have been reported previously to be resistant to the lethal effects of the *P. haemolytica* LKT (41). Our data suggest that the lack of biological activity of *P. haemolytica* LKT against nonruminant leukocytes reflects the inability of LKT to bind to these species of leukocytes.

These findings are significant in that there has not been a previous description of a specific binding site for *P. haemolytica* LKT, or other RTX toxins, on leukocytes. Most of the published reports on binding of LKT, or other RTX toxins, to cells have used biological activity (e.g., lysis) as their readout and did not focus on the binding event itself. However, Bauer and Welch recently reported the direct binding of *E. coli* hemolysin to sheep erythrocytes and concluded that it was calcium dependent (2). It is interesting to note that binding of *P. haemolytica* LKT to sheep erythrocytes was not observed in their system. In the present study, we detected LKT binding directly, without relying on a biological readout. However, our results with the anti-LKT MAb, and with porcine and human leukocytes, suggest that measuring the biological response to LKT might mirror binding.

Our data furthermore suggest that there is a protease K-sensitive molecule, presumably a protein, on the surface of the bovine leukocytes that is required for LKT binding. One might argue that this observation could reflect binding of protease K to the leukocyte membrane, where it could then degrade LKT. However, we believe this to be unlikely. Leukotoxic activity was eliminated by treatment with the same amount of protease K (0.1 mg per ml) used to treat the cells but was only partly reduced by incubation with lesser amounts (0.025 or 0.05 mg per ml). Using a casein hydrolysis assay, we estimated that 53 to 58% of the proteolytic activity added was in the supernatant of protease K-treated cells. We found that protease K-treated PMNs had approximately 20%, and that PBMCs had 10%, more proteolytic activity than control leukocytes in the casein digestion assay. Because 0.1 mg of protease K/ml was needed to completely eliminate LKT leukotoxic activity (there was little reduction with a two- or fourfold lesser amount of enzyme) it seems unlikely that the small amounts of protease K that might be adherent to the leukocytes could account for the nearly complete loss of LKT binding noted in our study. At this time, we cannot prove that the LKT binds directly to a protease K-sensitive protein. It is possible that the putative protein could be involved in some event secondary to the initial binding of the LKT. For example, perhaps some protein associates with the LKT, or stabilizes binding of the LKT to the cytoplasmic membrane, after the LKT first inserts itself into the lipid bilayer.

We have made efforts to identify a protein that is required for the binding of leukotoxin to the bovine leukocyte cell surface by performing ligand blot experiments with biotinylated LKT. Although we have observed a 43-kDa protein that associated with the blotted LKT (three separate experiments), we cannot consistently repeat this finding. Therefore, the identity of a receptor for the LKT, and any biological activity resulting from LKT binding, remains to be established. Identifying a binding site for the LKT would help us to better understand the diverse array of biological effects mediated by this toxin. It is commonly thought that the LKT and other RTX toxins act principally by inserting themselves into the lipid bilayer and forming pores that permit calcium influx and macromolecule efflux (3, 23, 28, 29, 32, 35, 46). However, there is other evidence that the interaction of RTX toxins with cells is more complex and that binding and some biological activities can be dissociated (9, 30, 31). This conclusion is supported by previous studies from our laboratory which showed that LKT activation of bovine leukocytes (i.e., release of reactive oxygen intermediates and degranulation) was blocked by verapamil (33), whereas the ability of the LKT to trigger apoptosis was unaffected (44). Because we have shown previously that verapamil greatly reduces Ca^{2+} uptake by LKT-treated bovine PMNs (33), these findings suggest that apoptosis, but not activation, might be less dependent on Ca^{2+} uptake. This observation is consistent with the hypothesis that more than one signal transduction pathway is responsible for the activating and lethal effects of the LKT for bovine leukocytes.

In summary, we have described a new method for detecting the binding of *P. haemolytica* LKT to bovine leukocytes and have obtained evidence that the biological activity of the LKT (i.e., cell lysis) is reflected in its ability to bind leukocytes. Our results suggest that nonruminant mammalian species of leukocytes (i.e., pig and human) do not bind LKT, thus explaining why they are resistant to killing by LKT. Our use of an LKT mutant strain of *P. haemolytica* helps us to exclude the possibility that the binding assay detected some other component of *P. haemolytica* that was present in the culture filtrates. The results of this study provide new insights into the interactions between *P. haemolytica* LKT and bovine leukocytes. Identifying what surface protein or other structures are involved in the

initial adherence of the LKT to the leukocyte cytoplasmic membrane will help us to better understand how LKT activates the biological responses for which it is noted (e.g., oxidative burst, degranulation, apoptosis). This information might yield new strategies for reducing the severity of, and minimizing the economic losses associated with, pulmonary pasteurellosis.

ACKNOWLEDGMENTS

This work was supported by funds from the USDA National Research Initiative (92-37204-7927, 93-37204-9204, and 96-35204-3666).

We thank the School of Veterinary Medicine word processing personnel for preparing the manuscript. We thank S. Srikuman (Lincoln, Nebr.) for the generous gift of MAb MM601 and G. Weinstock (Houston, Tex.) for providing *P. haemolytica* strains 59B049 and 59B0071. We thank Lawrence Wang for technical assistance and Steven Giles for his computer graphics assistance.

REFERENCES

1. **Baluyut, C. S., R. R. Simonson, W. J. Bemrick, and S. K. Maheswaran.** 1981. Interaction of *Pasteurella haemolytica* with bovine neutrophils: identification and partial characterization of a cytotoxin. Am. J. Vet. Res. **42:**1920–1926.
2. **Bauer, M. E., and R. A. Welch.** 1996. Association of RTX toxins with erythrocytes. Infect. Immun. **64:**4665–4672.
3. **Benz, R., A. Schmid, W. Wagner, and W. Goebel.** 1989. Pore formation by the *Escherichia coli* hemolysin: evidence for an association-dissociation equilibrium of the pore-forming aggregates. Infect. Immun. **57:**887–895.
4. **Bhakdi, S., and E. Martin.** 1991. Superoxide generation by human neutrophils induced by low doses of *Escherichia coli* hemolysin. Infect. Immun. **59:**2955–2962.
5. **Boehm, D. F., R. A. Welch, and I. S. Snyder.** 1990. Domains of *Escherichia coli* hemolysin (HlyA) involved in binding of calcium and erythrocyte membranes. Infect. Immun. **58:**1957–1964.
6. **Car, B. D., M. M. Suyemoto, N. R. Neilsen, and D. O. Slauson.** 1991. The role of leukocytes in the pathogenesis of fibrin deposition in bovine acute lung injury. Am. J. Pathol. **138:**1191–1197.
7. **Chidambaram, M., B. Sharma, S. F. Petras, C. P. Reese, S. Froshauer, and G. M. Weinstock.** 1995. Isolation of *Pasteurella haemolytica* leukotoxin mutants. Infect. Immun. **63:**1027–1032.
8. **Clinkenbeard, K. D., C. R. Clarke, C. M. Hague, P. Clinkenbeard, S. Srikumaran, and R. J. Morton.** 1994. *Pasteurella haemolytica* leukotoxin-induced synthesis of eicosanoids by bovine neutrophils in vitro. J. Leukocyte Biol. **56:**644–649.
9. **Clinkenbeard, K. D., D. A. Mosier, and A. W. Confer.** 1989. Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella haemolytica* leukotoxin. Infect. Immun. **57:**420–425.
10. **Confer, A. W., R. J. Panciera, K. D. Clinkenbeard, and D. A. Mosier.** 1990. Molecular aspects of virulence of *Pasteurella haemolytica*. Can. J. Vet. Res. **54:**S48–S52.
11. **Cruz, W. T., R. Young, Y. F. Chang, and D. K. Struck.** 1990. Deletion analysis resolves cell-binding and lytic domains of the *Pasteurella* leukotoxin. Mol. Microbiol. **4:**1933–1939.
12. **Czuprynski, C. J., and O. Ortiz-Carranza.** 1992. *Pasteurella haemolytica* leukotoxin inhibits mitogen-induced bovine peripheral blood mononuclear cell proliferation *in vitro*. Microb. Pathog. **12:**459–463.
13. **Czuprynski, C. J., E. J. Noel, O. Ortiz-Carranza, and S. Srikumaran.** 1991. Activation of bovine neutrophils by partially purified *Pasteurella haemolytica* leukotoxin. Infect. Immun. **59:**3126–3133.
14. **Dom, P., F. Haesebrouck, E. M. Kamp, and M. A. Smits.** 1994. NAD-independent *Actinobacillus pleuropneumoniae* strains: production of RTX toxins and interactions with porcine phagocytes. Vet. Microbiol. **39:**205–218.
15. **Eberspächer, B., F. Hugo, and S. Bhakdi.** 1989. Quantitative study of the binding and hemolytic efficiency of *Escherichia coli* hemolysin. Infect. Immun. **57:**983–988.
16. **Frank, G. H.** 1989. Pasteurellosis of cattle, p. 197–222. *In* C. Adlam and J. M. Rutter (ed.), Pasteurella and pasteurellosis. Academic Press, New York, N.Y.
17. **Gentry, J. J., and S. Srikumaran.** 1991. Neutralizing monoclonal antibodies to *Pasteurella haemolytica* leukotoxin affinity-purify the toxin from crude culture supernatants. Microb. Pathog. **10:**411–417.
18. **Gerbig, D. G., Jr., R. D. Walker, J. C. Baker, J. S. Foster, and R. N. Moore.** 1989. Calcium ion involvement in the action of *Pasteurella haemolytica* leukotoxin. Vet. Microbiol. **19:**325–335.
19. **Gerbig, D. G., Jr., M. R. Cameron, D. K. Struck, and R. N. Moore.** 1992. Characterization of a neutralizing monoclonal antibody to *Pasteurella haemolytica* leukotoxin. Infect. Immun. **60:**1734–1739.
20. **Henricks, P. A., G. J. Binkhorst, A. A. Drijver, and F. P. Nijkamp.** 1992. *Pasteurella haemolytica* leukotoxin enhances production of leukotriene B_4 and 5-hydroxyeicosatetraenoic acid by bovine polymorphonuclear leukocytes. Infect. Immun. **60:**3238–3243.
21. **Hughes, H. P. A., M. Campos, L. McDougall, T. K. Beskorwayne, A. A. Potter, and L. A. Babiuk.** 1994. Regulation of major histocompatibility complex class II expression by *Pasteurella haemolytica* leukotoxin. Infect. Immun. **62:**1609–1615.
22. **Ji, G. E., and P. O'Hanley.** 1990. Epitopes of *Escherichia coli* alpha-hemolysin: identification of monoclonal antibodies that prevent hemolysis. Infect. Immun. **58:**3029–3035.
23. **Konig, B., A. Ludwig, W. Goebel, and W. Konig.** 1994. Pore formation by the *Escherichia coli* alpha-hemolysin: role for mediator release from human inflammatory cells. Infect. Immun. **62:**4611–4617.
24. **Ludwig, A., T. Jarchau, R. Benz, and W. Goebel.** 1988. The repeat domain of *Escherichia coli* haemolysin (HlyA) is responsible for its Ca^{2+}-dependent binding to erythrocytes. Mol. Gen. Genet. **214:**553–561.
25. **Maheswaran, S. K., M. S. Kannan, D. J. Weiss, K. R. Reddy, E. L. Townsend, H. S. Yoo, B. W. Lee, and L. O. Whiteley.** 1993. Enhancement of neutrophil-mediated injury to bovine pulmonary endothelial cells by *Pasteurella haemolytica* leukotoxin. Infect. Immun. **61:**2618–2625.
26. **Maheswaran, S. K., D. J. Weiss, M. S. Kannan, E. L. Townsend, K. R. Reddy, L. O. Whiteley, and S. Srikumaran.** 1992. Effects of *Pasteurella haemolytica* A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals. Vet. Immunol. Immunopathol. **33:**51–68.
27. **Majury, A. L., and P. E. Shewen.** 1991. The effect of *Pasteurella haemolytica* A1 leukotoxic culture supernate on the in vitro proliferative response of bovine lymphocytes. Vet. Immunol. Immunopathol. **29:**41–56.
28. **Mangan, D. F., N. S. Taichman, E. T. Lally, and S. M. Wahl.** 1991. Lethal effects of *Actinobacillus actinomycetemcomitans* leukotoxin on human T lymphocytes. Infect. Immun. **59:**3267–3272.
29. **Menestrina, G., N. Mackman, I. B. Holland, and S. Bhakdi.** 1987. *Escherichia coli* haemolysin forms voltage-dependent ion channels in lipid membranes. Biochim. Biophys. Acta **905:**109–117.
30. **Menestrina, G., C. Moser, S. Pellet, and R. Welch.** 1994. Pore-formation by *Escherichia coli* hemolysis (HlyA) and other members of RTX toxins family. Toxicology **87:**249–267.
31. **Moayeri, M., and R. A. Welch.** 1994. Effects of temperature, time, and toxin concentration on lesion formation by the *Escherichia coli* hemolysin. Infect. Immun. **62:**4124–4134.
32. **Oropeza-Wekerle, R. L., S. Muller, J. P. Briand, R. Benz, A. Schmid, and W. Goebel.** 1992. Haemolysin-deprived synthetic peptides with pore-forming and haemolytic activity. Mol. Microbiol. **6:**115–121.
33. **Ortiz-Carranza, O., and C. J. Czuprynski.** 1992. Activation of bovine neutrophils by *Pasteurella haemolytica* leukotoxin is calcium dependent. J. Leukocyte Biol. **52:**558–564.
34. **Petras, S. F., M. Chidambaram, E. F. Illyes, S. Froshauer, G. M. Weinstock, and C. P. Reese.** 1995. Antigenic and virulence properties of *Pasteurella haemolytica* leukotoxin mutants. Infect. Immun. **63:**1033–1039.
35. **Rashid, J., D. J. Weiss, R. Bach, and M. Murtaugh.** 1997. Role of tissue factor in intra-alveolar fibrin deposition and coagulopathy associated with pneumonic pasteurellosis in cattle. Am. J. Vet. Res. **58:**28–33.
36. **Rogel, A., and E. Hanski.** 1992. Distinct steps in the penetration of adenylate cyclase toxin of *Bordetella pertussis* into sheep erythrocytes. J. Biol. Chem. **267:**22599–22605.
37. **Rowe, G. E., S. Pellett, and R. A. Welch.** 1994. Analysis of toxinogenic functions associated with the RTX repeat region and monoclonal antibody D12 epitope of *Escherichia coli* hemolysin. Infect. Immun. **62:**579–588.
38. **Sharma, S. A., T. W. J. Olchowy, and M. A. Brieder.** 1992. Alveolar macrophage and neutrophil interactions in *Pasteurella haemolytica*-induced endothelial cell injury. J. Infect. Dis. **165:**651–657.
39. **Shenker, B. J., L. A. Vitale, I. Keiba, G. Harrison, P. Berthold, E. Golub, and E. T. Lally.** 1994. Flow cytometric analysis of the cytotoxic effects of *Actinobacillus actinomycetemcomitans* leukotoxin on human natural killer cells. J. Leukocyte Biol. **55:**153–160.
40. **Shewen, P. E., and B. N. Wilkie.** 1985. Evidence for the *Pasteurella haemolytica* cytotoxin as a product of actively growing bacteria. Am. J. Vet. Res. **46:**1212–1214.
41. **Shewen, P. E., and B. N. Wilkie.** 1982. Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. Infect. Immun. **35:**91–94.
42. **Slocombe, R. F., J. Malark, R. Ingersoll, F. J. Derksen, and N. E. Robinson.** 1985. Importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis in calves. Am. J. Vet. Res. **46:**2253–2258.
43. **Stevens, P. K., and C. J. Czuprynski.** 1995. Dissociation of cytolysis and monokine release by bovine mononuclear phagocytes incubated with *Pasteurella haemolytica* partially-purified leukotoxin and lipopolysaccharide. Can. J. Vet. Res. **59:**110–117.
44. **Stevens, P. K., and C. J. Czuprynski.** 1996. *Pasteurella haemolytica* leukotoxin induces bovine leukocytes to undergo morphologic changes consistent with apoptosis in vitro. Infect. Immun. **64:**2687–2694.
45. **Styrt, B., R. D. Walker, L. D. Dahl, and A. Potter.** 1990. Time and temper-

ature dependence of granulocyte damage by leucotoxic supernatants from *Pasteurella haemolytica* A1. J. Gen. Microbiol. **136:**2173–2178.
46. **Taichman, N. S., I. M. Iwase, E. T. Lally, S. J. Shattil, M. E. Cunningham, and H. M. Korchak.** 1991. Early changes in cytosolic calcium and membrane potential induced by *Actinobacillus actinomycetemcomitans* leukotoxin in susceptible and resistant target cells. J. Immunol. **147:**3587–3594.
47. **Welch, R. A.** 1991. Pore-forming cytolysins of gram-negative bacteria. Mol. Microbiol. **5:**521–528.
48. **Yoo, H. S., B. S. Rajagopal, S. K. Maheswaran, and T. R. Ames.** 1995. Purified *Pasteurella haemolytica* leukotoxin induces expression of inflammatory cytokines from bovine alveolar macrophages. Microb. Pathog. **18:**237–252.

Editor: P. E. Orndorff