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Pasteurella haemolytica Leukotoxin Induces Bovine Leukocytes To Undergo Morphologic Changes Consistent with Apoptosis In Vitro

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Infection of the bovine lung with *Pasteurella haemolytica* results in an acute respiratory disorder known as pneumonic pasteurellosis. One of the key virulence determinants used by this bacterium is secretion of an exotoxin that is specific for ruminant leukocytes (leukotoxin). At low concentrations, the leukotoxin can activate ruminant leukocytes, whereas at higher concentrations, it inhibits leukocyte functions and is cytolytic, presumably as a result of pore formation and subsequent membrane permeabilization. We have investigated the possibility that the activation-inhibition paradox is explained in part by leukotoxin-mediated apoptosis (i.e., activation-induced cell death) of bovine leukocytes. Incubation of bovine leukocytes with *P. haemolytica* leukotoxin caused marked cytoplasmic membrane blebbing (zeiosis) and chromatin condensation and margination, both of which are hallmarks of apoptosis. The observed morphologic changes in bovine leukocytes were leukotoxin dependent, because they were significantly diminished in the presence of an anti-leukotoxin monoclonal antibody. In addition, bovine leukocytes incubated with culture supernatant from a mutant strain of *P. haemolytica* that does not produce any detectable leukotoxin failed to exhibit the morphologic changes characteristic of cells undergoing apoptosis. These observations may represent an important mechanism by which *P. haemolytica* overwhelms host defenses, contributing to the fibrinous pleuropneumonia characteristic of bovine pasteurellosis.

Bovine pneumonic pasteurellosis is an acute infection with *Pasteurella haemolytica* which often results in a severe fibrinous pleuropneumonia. *P. haemolytica* produces several virulence factors that enable it to evade host defenses. Although they all play a role in the pathogenesis of the disease, one of them, a leukotoxin (LKT) that can specifically kill ruminant leukocytes (1, 26, 55) and platelets (12), is probably the most important virulence factor. This conclusion is supported by the finding that calves challenged with wild-type *P. haemolytica* had substantially greater mortality and lung lesion scores than did calves challenged with a mutant strain of *P. haemolytica* that was completely deficient in the production of LKT but not other virulence factors (47).

The LKT is a 104-kDa protein that is synthesized and secreted by *P. haemolytica* during logarithmic-phase growth (8, 55). The LKT is a member of a broadly distributed family of exoproteins called RTX toxins (62), which are produced by a variety of gram-negative bacteria. Other members of the RTX family include the *Escherichia coli* hemolysin, the *Bordetella pertussis* adenylate cyclase/hemolysin, the LKT of *Actinobacillus actinomycetemcomitans*, the cytotoxins secreted by *A. pleuropneumoniae* and *A. suis*, and the hemolysins produced by *Proteus vulgaris* and *Morganella morganii* (62). All the members of this family share a tandemly repeated glycine-rich sequence of nine amino acids that binds calcium and justifies the designation RTX (for repeats in toxin) (62). The cytolytic activity of RTX toxins is calcium dependent (6, 23, 38). RTX toxins are thought to lyse target cells by the formation of transmembrane pores that render the cytoplasmic membrane permeable to ion and water flux, resulting in cell lysis (4, 11, 43). Many RTX toxins, including the *P. haemolytica* LKT, are also potent stimuli for leukocytes when they are present at low concentrations (5, 16, 18, 20, 24, 33, 34, 40, 58). It has been suggested that many of the abnormalities typical of pasteurellosis are mediated by activated leukocytes that release reactive oxygen intermediates, or other inflammatory mediators, in a poorly controlled manner (39, 52). The mechanism for LKT-mediated leukocyte activation has not been fully determined, but an increased intracellular Ca^{2+} concentration is required (45).

Apoptosis is a process of cell death that can be distinguished from necrosis by various morphological and biochemical criteria (30, 51, 63). These include chromatin collapse with subsequent chromatin margination in crescent-shaped masses around the periphery of the nucleus, blebbing of the cytoplasmic membrane (i.e., zeiosis), and internucleosomal cleavage of DNA into nucleosome-sized fragments. The process of apoptosis is essential, playing a major role in normal homeostasis and development. Since leukocytes have the ability to undergo apoptosis, it is not surprising that some pathogenic bacteria attempt to utilize apoptosis to their advantage. Recently, several laboratories have reported that certain RTX toxins can induce target cells to undergo apoptosis (31, 42, 53). The ability of low concentrations of bacterial toxins to induce cell death in leukocytes may play a significant role in initiation and persistence of infection, giving the pathogen a distinct advantage in the battle with host defense mechanisms. The purpose of this investigation was to determine if the *P. haemolytica* LKT induces apoptosis in bovine leukocytes.

MATERIALS AND METHODS

Leukocyte isolation. Alveolar macrophages were obtained as described previously (58). Briefly, freshly excised lungs from healthy Holstein cattle were lavaged with 2.0 liters of phosphate-buffered saline (pH 7.4) with 10 mM EDTA. The lavage fluid was decanted and kept on ice for 40 min before being passed through a 40-mm-mesh nylon filter (Falcon, Schiller Park, Ill.). The filtrate was washed three times at 4°C (for 10 min at 200 × *g*), and the pellet was resuspended in 50 ml of cold Hanks balanced salt solution (HBSS; Sigma Chemical Co., St. Louis, Mo.). Contaminating erythrocytes were lysed by hypotonic shock (45 s with 13.2 mM phosphate buffer without saline before restoring isotonicity), and the leukocytes were resuspended at 10^7 cells per ml in RPMI 1640 (Gibco, Grand Island, N.Y.)–5% fetal bovine serum (FBS; Intergen, Purchase, N.Y.).

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Alveolar macrophages accounted for more than 90% of the suspended cells, as determined by microscopic evaluation of Diff-Quik-stained cytocentrifuge smears.

Peripheral blood was collected from healthy Holstein donor cows into 20-ml evacuated glass tubes (Becton-Dickinson, Rutherford, N.J.) containing 2.0 ml of 4% sodium citrate 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. To obtain lymphocytes and monocytes, the buffy coat cells were diluted 1:4 (vol/vol) in HBSS with 10 mM EDTA and overlaid on Nycoprep gradients (density, 1.077; Nycomed, Oslo, Norway). These were centrifuged at room temperature (350 × *g*) for 30 min, and the lymphocyte- and monocyte-containing fractions were collected separately. These fractions were washed four times at 4°C in HBSS and resuspended in RPMI 1640–5% fetal bovine serum at 1.0×10^7 cells per ml. The monocyte-containing fraction typically consisted of greater than 80% monocytes as determined by esterase staining (7, 35), and the lymphocyte-enriched suspension contained approximately 70% lymphocytes. Neutrophils were obtained as described previously (15) by flash hypotonic lysis of blood and centrifugation through a Percoll gradient (Pharmacia, Uppsala, Sweden). The neutrophil pellets were washed twice in HBSS and resuspended at 1.0×10^7 cells per ml in HBSS or in RPMI 1640–5% fetal bovine serum. The neutrophil suspensions were greater than 95% pure as determined by evaluation of Diff-Quik-stained cytocentrifuge smears and greater than 95% viable as estimated by trypan blue exclusion.

P. haemolytica. Three strains of *P. haemolytica* were used in the study. The first of these, originally isolated from a pneumonic lung of a feedlot cow, was obtained from R. Corstvet, Baton Rouge, La. The other two strains were obtained from G. Weinstock, Houston, Tex. The first of these (strain 59B409) was a wild-type strain isolated from a pneumonic bovine lung. This parent strain was mutagenized with nitrosoguanidine to obtain an LKT mutant (59B0071) that lacked detectable LKT but was unaltered in its rate of growth, antibiotic susceptibility, and production of other known virulence determinants (9). Consistent with its lack of LKT production, mutant strain 59B0071 exhibited reduced virulence for experimentally infected calves (47).

LKT production. All LKT preparations were produced and partially purified as described previously (57). Briefly, *P. haemolytica* A1 was inoculated onto blood agar (Remel, Lenexa, Kans.) and incubated overnight 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 in rotating 15-ml polypropylene tubes. For each bacterial strain, a 2.0-ml aliquot of this suspension was then used to inoculate 48 ml of BHI-YE in 250-ml Erlenmeyer flasks. The flasks were then incubated for 2 h at 37°C with gentle shaking. The bacteria were then collected by centrifugation (1,600 × *g* for 15 min) and resuspended in 100 ml of RPMI 1640 supplemented with L-glutamine (4.0 mM). These suspensions were transferred to 500-ml Erlenmeyer flasks and incubated for 2 h at 37°C with moderate shaking. The bacteria were harvested by centrifugation (1,600 × *g* for 20 min), and the crude LKT-containing supernatant was collected with a pipette and passed through a 0.45-mm bottletop filter (Nalgene, Rochester, N.Y.). Aliquots (20 ml) of crude LKT were diluted to 180 ml with RPMI 1640 in an Amicon ultrafiltration unit equipped with a 62-mm diameter YM-100 ultrafiltration membrane. The volume was then reduced to 20 ml over a 30-min period by applying transmembrane pressure (8 lb/in^2) with nitrogen gas. The partially purified LKT preparations that remained were then collected and stored as 1.0-ml aliquots at −70°C. LKT prepared from the strain of *P. haemolytica* obtained from R. Corstvet was designated LKT (C); LKT prepared from the parental strain 59B049, obtained from G. Weinstock, is referred to as LKT (W); and LKT derived from the LKT-deficient mutant strain 59B0071 (derived from strain 59B409) is designated LKT (−).

LKT-neutralizing MAb. A murine monoclonal antibody (MAb) (immunoglobulin G1) that neutralizes the cytolytic effects of *P. haemolytica* LKT was generously provided by S. Srikumaran, Lincoln, Nebr. Preparation and use of this MAb (mm601) have been described previously (22).

LKT activity. The cytolytic activity of the LKT preparations was determined for each experiment by incubating serial dilutions of the partially purified LKT preparations with 10^6 bovine neutrophils in 1.0 ml of HBSS in 1.8-ml polypropylene tubes at 39°C for 1 h with constant rotation. One unit of LKT activity is defined as the LKT dilution that kills 50% of bovine neutrophils within 1 h, as assessed by failure to exclude trypan blue. The LKT (−) preparation culture filtrates, which lacked cytolytic activity, were subjected to the same preparative procedures and diluted to the same extent as was the parental LKT (W) preparation.

Apoptosis assays. (i) Zeiosis. Zeiosis was determined by incubating 10^6 neutrophils in 1.0 ml of HBSS with various treatments at 39°C for 1 h. The cells were then fixed with 2% paraformaldehyde and examined by light microscopy (magnification, ×400). For every test condition, 100 to 200 cells were counted, and they were scored positive if the cell membrane exhibited deformation (blebbing) or marked shape change and were scored negative if they remained spherical.

(ii) Chromatin condensation. To quantify the margination and condensation of chromatin material, 10^6 bovine alveolar macrophages, lymphocytes, monocytes, or neutrophils were incubated at 39°C in 24-well tissue culture plates (Falcon) that contained 12-mm-diameter glass coverslips that had been coated with poly-L-lysine (Sigma Chemical Co.). Alveolar macrophages and neutrophils were incubated for 2 h and lymphocytes and monocytes were incubated for 4 h before being fixed with 4% paraformaldehyde. These times were used because preliminary experiments indicated that they resulted in optimal chromatin margination responses upon exposure to LKT. The fixed cells were stored for less than 24 h at 4°C until stained. To stain the chromatin material, the fixed cells were incubated for 10 min at room temperature with 30 mg of Hoescht dye 33342 (Sigma Chemical Co.) per ml and counterstained for 15 min at room temperature with 0.1% trypan blue in phosphate-buffered saline (PBS)–0.1% Triton X-100. The stained coverslips were washed five times with PBS before being mounted on glass slides with Crytal/Mount (Biomeda, Foster City, Calif.). The chromatin samples of more than 100 cells per test condition were individually examined by UV microscopy. If the chromatin was sharply condensed and pycnotic or was distributed along the margins of the nuclear envelope, the cell was considered positive. If the nuclear material remained uncondensed and exhibited normal morphology, the cells were scored as negative.

Apoptotic index. For all data, an apoptotic index (AI) was calculated from the following formula: $100 \times (A - B)/(100 - B) = \text{AI}$, where A is the percentage of apoptotic cells for that particular assay and B is the percentage of background apoptotic cells as measured from the negative controls. Staurosporine (200 nM; Sigma Chemical Co.) was used as a positive control in most experiments, because it has been shown previously to induce apoptosis in many cell types (2).

Statistical analysis. Data were analyzed by a one-way analysis of variance, followed by the Student *t* test, as performed with the INSTAT statistical computer program (GraphPad Software, Inc., San Diego, Calif.).

RESULTS

***P. haemolytica* LKT induces zeiosis in bovine neutrophil plasma membranes.** Disturbances in the neutrophil plasma membranes, consistent with the structural changes associated with apoptosis (13), were observed following a 1-h incubation with low concentrations of LKT (Fig. 1). LKT concentrations of 0.1 to 1.0 U of LKT activity caused significant membrane blebbing (Fig. 2), with the observed effects being most pronounced at 0.5 U of LKT activity.

To exclude the possibility that some other component in our partially purified LKT preparation was responsible for zeiosis, bovine neutrophils were incubated at 39°C with 0.5 U of LKT from two different LKT-producing strains of *P. haemolytica* [LKT (C) and LKT (W)] and an equivalent amount of culture filtrate from an LKT-deficient mutant of *P. haemolytica* [LKT (−)]. Controls included untreated neutrophils, neutrophils incubated with LKT (C) that had been preincubated with a neutralizing anti-LKT MAb (mm601), and neutrophils incubated with 200 nM staurosporine as a positive control for apoptosis. Staurosporine was chosen on the basis of reports of its ability to induce apoptosis in various cell types (3) and after preliminary experiments revealed that it consistently caused apoptotic changes in bovine leukocytes. After incubation, the neutrophils were fixed with paraformaldehyde, and more than 200 cells per test group were individually examined by phase-contrast light microscopy for evidence of zeiosis. The results indicate that exposure to both LKT (C) and LKT (W) significantly induced zeiosis in bovine neutrophils (Fig. 3). In contrast, incubation of bovine neutrophils with LKT (−) failed to induce these morphologic changes. Preincubation of LKT (C) with anti-LKT MAb mm601 (final dilution, 1:1,000 [vol/vol]) dramatically blocked zeiosis in the bovine neutrophil membranes. These data indicate that biologically active LKT is required for the observed alterations in the bovine neutrophil plasma membrane.

***P. haemolytica* LKT induces the formation of pycnotic nuclei in bovine leukocytes.** Nuclear collapse is a hallmark of cells undergoing apoptosis (13). By using DNA-binding dyes, this process can be visualized as extremely condensed chromatin (i.e., pycnotic nuclei) that marginates in a crescent or horseshoe shape around the nuclear envelope or completely collapse into one or more spheres (Fig. 4). A significant increase in chromatin condensation and margination was observed in bovine alveolar macrophages, peripheral blood neutrophils, and

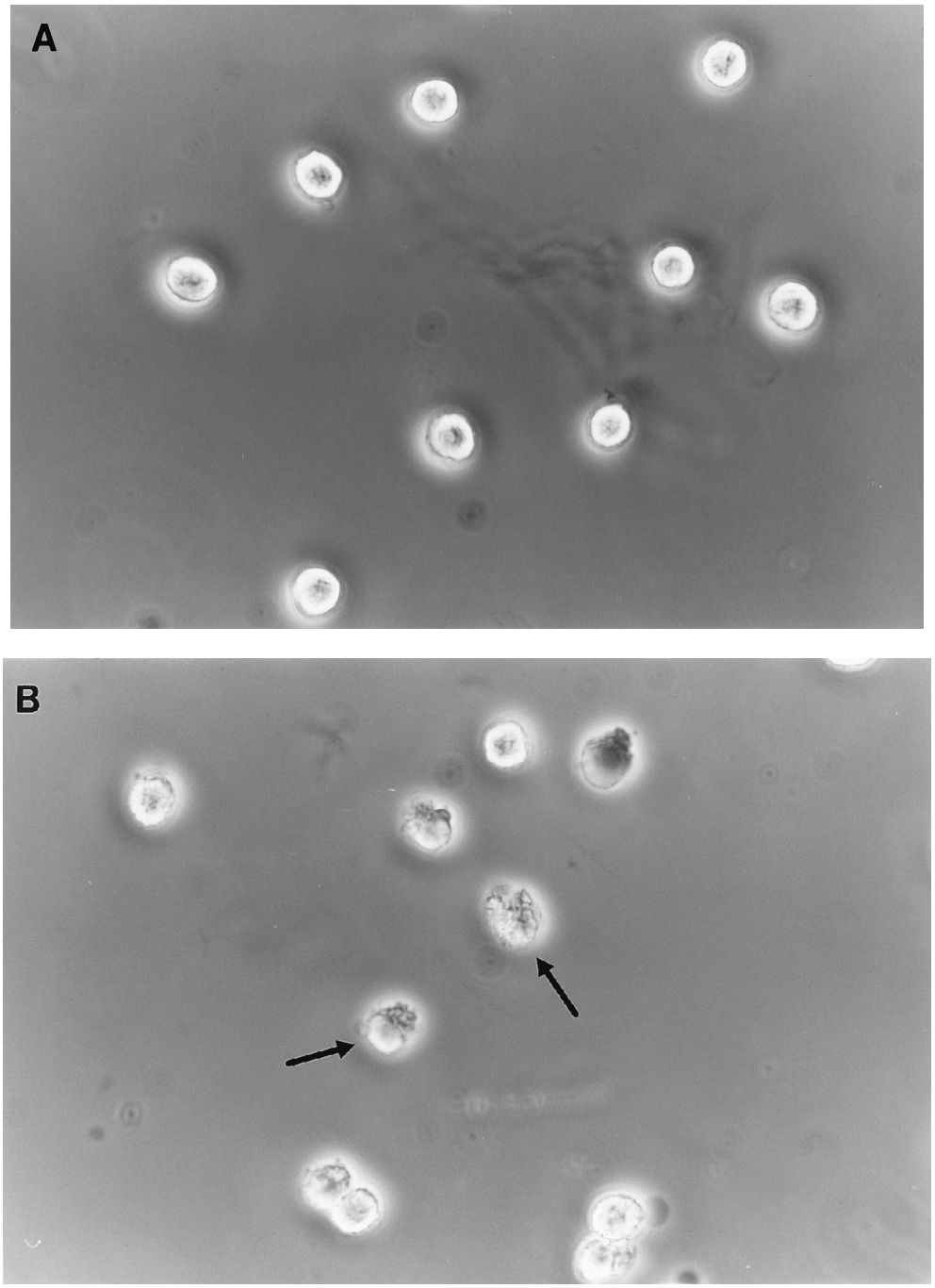

FIG. 1. Phase-contrast photomicrographs of LKT-induced zeiosis in bovine peripheral blood neutrophils. (A) Neutrophils incubated in medium alone for 1 h at 39°C prior to microscopic examination exhibit the round morphology typical of healthy neutrophils. (B) Neutrophils incubated for 1 h at 39°C with 0.5 U of LKT exhibit severe membrane deformation (zeiosis) typical of cells undergoing apoptosis (arrows). Magnification, ×400.

peripheral blood monocytes after incubation with 0.5 U of LKT (C) or LKT (W) (Fig. 5). Bovine lymphocytes also exhibited a significant increase in chromatin condensation after incubation with LKT (C). In contrast, incubation of bovine leukocytes with LKT (−) or with 0.5 U of LKT (C) that had been preincubated with MAb mm601 failed to induce any significant changes in the chromatin distribution of these cells. These data suggest that the *P. haemolytica* LKT, and not other bacterial components, is principally required for inducing apoptotic changes in bovine leukocyte chromatin distribution.

Verapamil does not block LKT-mediated zeiosis in bovine neutrophils. Increased intracellular calcium concentration has been associated with apoptosis in some but not all cell types (2, 21, 31, 46, 59). Because the leukocyte-activating activity of the *P. haemolytica* LKT is Ca^{2+} dependent (23, 45), we decided to determine if LKT-mediated apoptosis of bovine leukocytes was also Ca^{2+} dependent. To do this, bovine neutrophils were incubated with LKT in the absence or presence of verapamil, a membrane Ca^{2+} channel blocker (36) that has been shown previously to inhibit LKT-mediated Ca^{2+} influx and LKT-me-

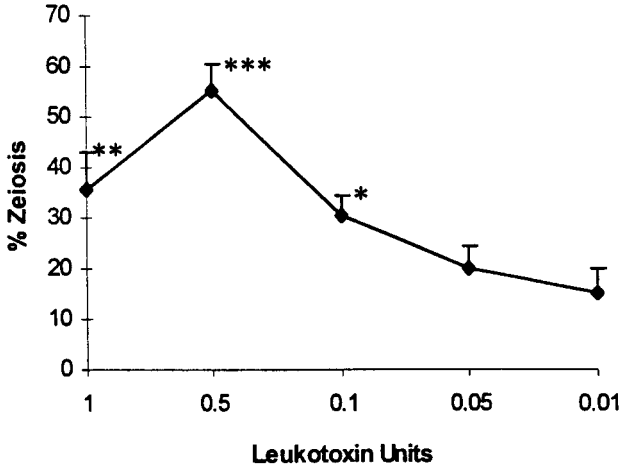

FIG. 2. Dose-dependent LKT-induced alterations in bovine neutrophil morphology (i.e., zeiosis). Neutrophils (10^6 cells) in 1.0 ml of RPMI 1640–5% FBS were incubated at 39°C with various concentrations of LKT for 1 h before being examined by phase-contrast light microscopy (magnification, ×400). Significant changes in the neutrophil plasma membrane, consistent with cells undergoing apoptosis, were observed for cells treated with between 0.1 and 1.0 U of LKT. Zeiosis for negative control neutrophils was 9.1 ± 2.4. Results shown are the mean and standard error of the mean for five separate experiments with neutrophils prepared from different donors. Symbols: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

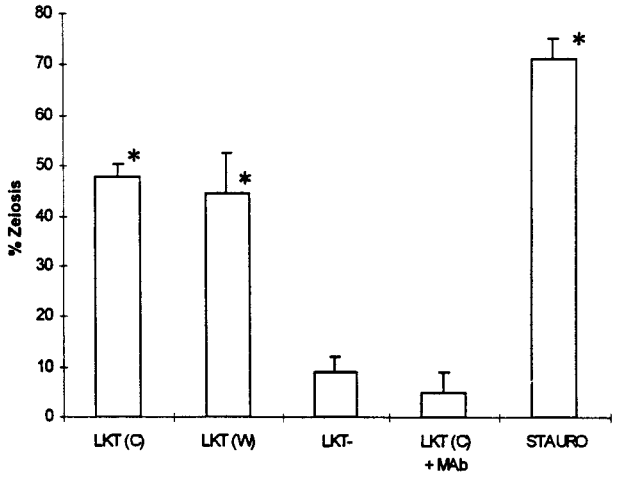

FIG. 3. Zeiosis does not occur in the absence of biologically active LKT. Neutrophils (10^6 cells) in 1.0 ml of RPMI 1640–5% FBS were incubated with 0.5 U of LKT (C), 0.5 U of LKT (W), an equivalent amount of culture filtrate from LKT (−), or 0.5 U of LKT (C) that had been preincubated with a neutralizing anti-LKT MAb, mm601. Staurosporine (200 nM) (STAURO) was used as a positive control for apoptosis. After a 1-h incubation at 39°C, the cells were fixed with paraformaldehyde and examined by phase-contrast light microscopy for membrane blebbing. Staurosporine, LKT (C), and LKT (W) significantly induced zeiosis in the neutrophils, as did the staurosporine control. The LKT (−) and LKT (C) plus MAb treatment groups did not differ significantly from untreated control neutrophils, although both were significantly different from LKT (C). This indicates that the LKT, and not other bacterial products which may be present in our partially purified LKT preparations, was required for the observed morphologic changes in bovine neutrophils. The results shown are the mean ± standard error of the mean for five separate experiments with neutrophils obtained from different donors. *, $P < 0.05$.

diated neutrophil activation (23, 45). In contrast to our previous report, verapamil did not significantly inhibit LKT-mediated apoptosis in the present study (Fig. 6). This suggests that the mechanism for LKT-mediated apoptosis is not directly associated with the rapid influx of Ca^{2+}.

Propranolol inhibits LKT-mediated zeiosis in bovine neutrophils. Propranolol, a β-adrenergic antagonist (37), is incorporated into mammalian cell membranes, where it exerts a membrane stabilization effect (44). Propranolol can also inhibit Ca^{2+} current and Ca^{2+} uptake (50) and can block calcium ionophore-induced platelet aggregation (61). Because we reported previously that propranolol blocked LKT-mediated activation of bovine neutrophils (45), we investigated its effects on LKT-mediated apoptosis. Bovine neutrophils were incubated with LKT in the absence or presence of propranolol, fixed with paraformaldehyde, and examined by phase-contrast light microscopy for membrane blebbing. Significant inhibition of LKT-mediated apoptosis was observed in neutrophils treated with 100 μM but not 10 μM propranolol (Fig. 7). Our previous studies indicated that 10 μM propranolol was sufficient to prevent LKT-mediated Ca^{2+} uptake on the neutrophil activator. Thus, we conclude that the effect of propranolol in the present study may have been by a mechanism other than prevention of Ca^{2+} uptake.

DISCUSSION

The *P. haemolytica* LKT is well recognized for its ability to selectively kill ruminant leukocytes (1, 26, 55). The mechanism for LKT-mediated cell lysis, as for other RTX toxins, is thought to occur through the formation of transmembrane pores that render the target cell membrane permeable to ion and water flux (4, 11, 43). Although the ability of LKT to selectively destroy defending leukocytes represents an obvious advantage for virulent *P. haemolytica*, the activation of host leukocytes by low concentrations of LKT may be of even greater importance in the pathogenesis of pasteurellosis. Low concentrations of LKT (20 to 75 ng/ml) can induce bovine neutrophils to undergo a respiratory burst, release secondary granule constituents (16, 40), and secrete the inflammatory mediators 5-hydroxyeicosatetraenoic acid and leukotriene B_4 in a dose-dependent manner (10, 25). The modulatory effects of the LKT and other *P. haemolytica* virulence factors on bovine inflammatory cells probably contributes to the pathophysiology of bovine pasteurellosis (14). For example, the importance of neutrophils in the pathogenesis of pneumonic pasteurellosis was demonstrated by Slocombe et al. (56), who found that depletion of peripheral blood neutrophils by hydroxyurea administration effectively blocked the severe pathologic changes that were observed in control calves inoculated with *P. haemolytica*. Sharma et al. (52) demonstrated in an in vitro coculture system that alveolar macrophages and neutrophils can enhance the damage to endothelial cells incubated with live *P. haemolytica*. Likewise, Maheswaran et al. (39) demonstrated that LKT augmented the neutrophil-mediated killing of bovine pulmonary endothelial cells in vitro by stimulating neutrophil release of reactive oxygen intermediates.

LKT has also been reported to modulate the activity of bovine mononuclear cells. Exposure of bovine peripheral blood monocytes to LKT induces a marked downregulation of major histocompatibility complex class II expression and an impaired ability to present antigen (28). The in vitro proliferative response of bovine peripheral blood mononuclear cells to mitogen is also inhibited by exposure of these cells to low concentrations (approximately 35 ng/ml) of LKT (17, 41). In contrast to these inhibitory effects, bovine mononuclear phagocytes release the potent proinflammatory cytokines tumor necrosis factor and interleukin-1 after incubation with LKT. In one study, this LKT effect could not be completely dissociated

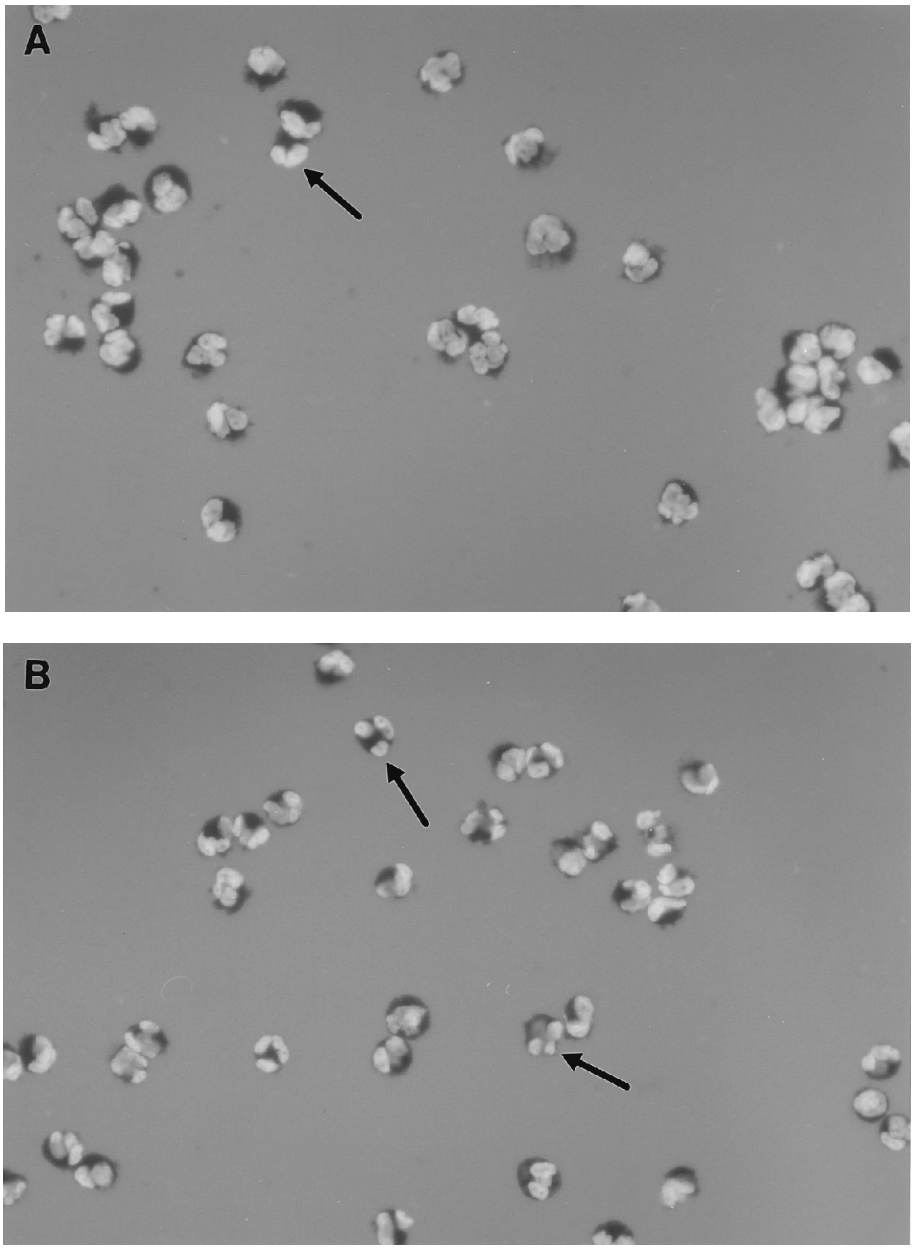

FIG. 4. Photomicrographs of peripheral blood neutrophils stained with 30 mg of Hoescht 33342 per ml to delineate nuclear morphology, as described in Materials and Methods. (A) Neutrophils incubated for 2 h at 39°C in RPMI 1640–5% FBS exhibit a normal nuclear morphology. Occasional cells demonstrate chromatin condensation (arrow). (B) Neutrophils incubated for 2 h with 0.5 U of LKT exhibit chromatin margination (arrows) consistent with cells that are undergoing apoptosis.

from lipopolysaccharide (57), whereas other investigators observed a similar response when bovine monocytes were incubated with LKT that appeared to be free of detectable lipopolysaccharide (60). The mechanism by which the LKT causes these stimulatory or suppressive effects has not yet been determined.

In this study, we have demonstrated that low concentrations of the *P. haemolytica* LKT (approximately 75 ng/ml) can induce bovine leukocytes to undergo apoptosis by using the accepted criteria of zeiosis, chromatin condensation, and nuclear fragmentation (30, 51, 63). We did not observe DNA degradation products ("DNA ladder") by agarose gel electrophoresis examination of LKT-treated neutrophils (data not shown). However, there are other examples in which apoptosis does not result in a DNA ladder and other instances in which visualization of a DNA ladder is not indicative of apoptosis (13). Several other investigators have reported that certain RTX toxins may induce target cells to undergo apoptosis, suggesting that this is a common virulence strategy among bacteria that produce these toxins. Shenker et al. (53) reported that the LKT of *A. actinomycetemcomitans* induced apoptosis in human natural killer cells and monocytes. Similarly, both $CD4^+$ and $CD8^+$ human lymphocytes were shown to undergo apoptosis after incubation with the *A. actinomycetemcomitans* LKT (42).

It is interesting that both the *P. haemolytica* and *A. actinomycetemcomitans* LKTs induce apoptosis in their target cells, as these RTX toxins exhibit a narrow range of target cell activity (ruminant and primate, respectively) (62). It may be

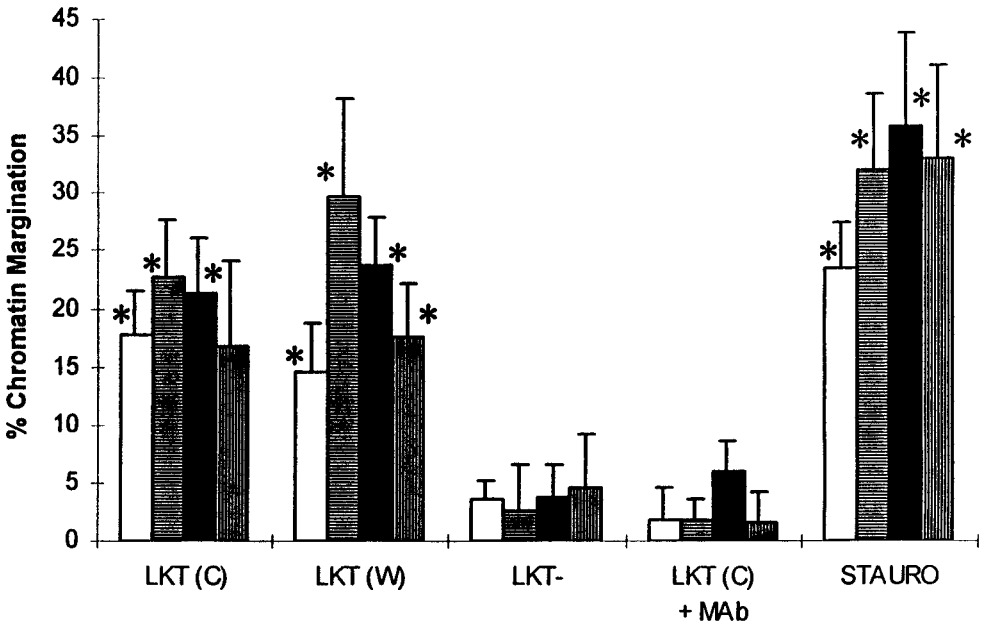

FIG. 5. Comparison of LKT-induced chromatin margination in bovine neutrophils, alveolar macrophages, monocytes, and lymphocytes. Bovine alveolar macrophages (□), lymphocytes (▤), monocytes (■), or neutrophils (▥) (10^6 cells) were incubated at 39°C with 0.5 U of LKT (C), 0.5 U of LKT (W), an equivalent amount of culture filtrate of LKT (−), or 0.5 U of LKT (C) that had been preincubated with a neutralizing anti-LKT MAb, mm601. Staurosporine (200 nM) (STAURO) was included as a positive control for apoptosis. After a 2-h (alveolar macrophages and neutrophils) or 4-h (lymphocytes and monocytes) incubation, the leukocytes were fixed with 4% paraformaldehyde and stained with 30 mg of Hoescht 33342 per ml to delineate nuclear morphology. The apoptotic index (AI) was calculated as described in Materials and Methods. LKT (C) and LKT (W) both significantly induced chromatin margination and condensation in all types of leukocytes. Neither the LKT mutant, LKT (−), nor LKT (C) incubated with a neutralizing anti-LKT MAb induced apoptosis in any of the leukocyte populations. The results shown are the mean and standard error of the mean for six (alveolar macrophages and neutrophils) or five (lymphocytes and monocytes) separate experiments with cells obtained from different donors. *, $P < 0.05$.

that the target cell specificity is mediated by a membrane "receptor" which, when bound to the toxin ligand, induces apoptosis. At higher toxin concentrations, the previously described process of osmotic lysis may kill target cells faster than the apoptotic death pathway does. Few other RTX toxins have been examined for their ability to induce apoptosis in host cells. The *E. coli* hemolysin, which exhibits a broad range of target cell activity, was reported not to induce human T lymphocytes cells to undergo apoptosis (29). However, the adenylate cyclase hemolysin of *B. pertussis*, the causative agent of whooping cough, induced apoptosis in a monocyte-macrophage tumor cell line (31). This toxin, which also has a broad range of activity (62), is interesting because the hemolysin portion of the molecule appears to facilitate cytosolic entry of the adenylate cyclase portion of the toxin molecule (48). Induction of apoptosis may be a function of this adenylate cyclase activity, because increased levels of cyclic AMP are known to induce apoptosis in some cells (19). Continued research into the ability of other RTX toxins to induce apoptosis may yield

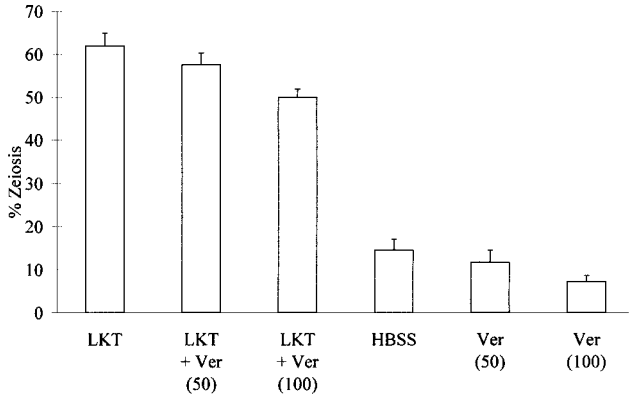

FIG. 6. Verapamil (Ver) does not inhibit LKT-mediated zeiosis in bovine neutrophils. Neutrophils (10^6 cells) were incubated in 1.0 ml of HBSS–5% FBS–0.5 U of LKT in the absence or presence of 10 or 100 μM verapamil (concentrations indicated in parentheses). After a 1-h incubation at 39°C, the cells were fixed with 4% paraformaldehyde and examined by phase-contrast light microscopy for membrane blebbing. The results shown are the mean ± standard error of the mean for five separate experiments.

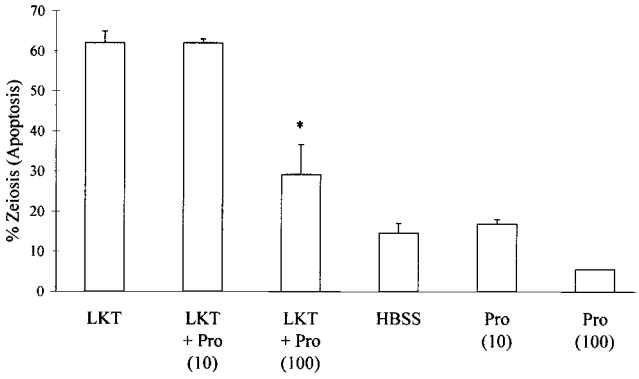

FIG. 7. Propranolol (Pro) partially inhibits LKT-mediated zeiosis in bovine neutrophils. Neutrophils (10^6 cells) were incubated in 1.0 ml of HBSS–5% FBS–0.5 U of LKT in the absence or presence of 10 or 100 μM propranolol (concentrations indicated in parentheses). After a 1-h incubation at 39°C, the cells were fixed with 4% paraformaldehyde and examined by phase-contrast light microscopy for membrane blebbing. The results shown are the mean ± standard error of the mean for five separate experiments. *, $P < 0.01$.

insights into the mechanisms of RTX toxin-mediated cytotoxicity.

The role of Ca^{2+} in apoptosis remains controversial. Several investigators have reported that the cellular changes associated with apoptosis are regulated by elevations in the cytosolic Ca^{2+} concentration. For example, Oshimi and Miyazaki (46) observed that a sustained rise in the intracellular calcium concentration was required for Fas antigen-mediated apoptosis in a human B-cell line. Increasing intracellular Ca^{2+} concentrations by exposure to thapsigargin, which increases the cytosolic Ca^{2+} concentration by inhibiting the ATP-dependent endoplasmic reticulum Ca^{2+} pump, or by addition of the Ca^{2+} ionophore A23187 also induces apoptosis in many cell types (21, 59). On the other hand, other researchers have found that increased intracellular Ca^{2+} concentrations are not associated with apoptosis. Murine thymocytes exposed to dexamethasone undergo apoptosis without any change in the intracellular Ca^{2+} concentrations (2). Kluck et al. (32) found that calcium chelation can induce apoptosis in several human cell lines. It may be that there are both Ca^{2+}-dependent and Ca^{2+}-independent pathways for apoptotic death or, as suggested by Kluck et al. (32), that apoptosis can result from impaired Ca^{2+} homeostasis.

In the present study, we indirectly examined the role of extracellular Ca^{2+} in LKT-mediated apoptosis of bovine neutrophils. Although calcium uptake is required for LKT-mediated neutrophil activation (45), it does not appear to be required for LKT-induced apoptotic cell death. The observation that verapamil does not inhibit LKT-induced apoptosis is particularly interesting, because it implies that the action of the LKT may be more complicated than formation of a simple pore that facilitates calcium influx into the cell. Perhaps the *P. haemolytica* LKT interacts with target cells in two ways: first, via membrane insertion and pore formation typical of RTX toxins, and second, with an as yet unidentified ruminant leukocyte-specific membrane constituent that activates a signal transduction pathway responsible for activation of the apoptotic death program. This hypothetical receptor would also help explain the species and cell type specificity of the *P. haemolytica* LKT (54). Propranolol inhibited LKT-mediated apoptosis but only at the highest concentration tested (100 μM). This is interesting, because a previous report from our laboratory demonstrated that 10 μM propranolol was sufficient to inhibit Ca^{2+} influx and the subsequent oxidative burst in bovine neutrophils exposed to LKT (45). The inhibitory effect of propranolol in the present study may reflect actions other than blockade of Ca^{2+} current and uptake, such as membrane stabilization in the treated neutrophils.

Secretion of an LKT that can induce apoptosis in host defense cells offers several potential advantages to pathogenic bacteria. The most obvious is that these LKTs will eventually kill leukocytes at low concentrations previously believed to be sublethal. This enhanced potency may play a significant role during the early stages of an infection such as pasteurellosis. In this scenario, inflammatory cells recruited to the lung will encounter small amounts of LKT that concomitantly stimulate an inflammatory response while dooming the leukocyte to progress along the pathway to apoptotic death. Meanwhile, the bacteria will multiply and increase the severity of the infection. Perhaps of greater importance than simply killing leukocytes is that apoptosis is a process that takes several hours to complete (51, 63). During this time, the doomed leukocytes may first be activated to release inflammatory mediators such as cytokines, oxygen radicals, granule constituents, and eicosanoids (5, 10, 16, 24, 25, 39, 57, 60) until their demise. The release of inflammatory mediators by macrophages undergoing apoptosis presents a particularly insidious threat, because it will result in a poorly controlled inflammatory response that will contribute to the intense inflammatory process that characterizes pulmonary pasteurellosis. There is some precedence for this, because murine macrophages undergoing apoptosis as a result of *Shigella flexneri* infection have been shown to release interleukin-1 in vitro (64). This is further supported by the work of others, who found that interleukin-1 could be processed and released by apoptotic monocytes and macrophages (27, 49).

In summary, we report here that exposure to low concentrations of *P. haemolytica* LKT in vitro induced bovine leukocytes to undergo morphologic changes consistent with apoptosis. Failure to observe apoptosis when an anti-LKT MAb was added or upon exposure to a culture filtrate from a non-LKT-producing strain of *P. haemolytica* suggests that the LKT, and not other bacterial virulence factors such as lipopolysaccharide, was required for induction of apoptosis. This finding suggests a novel mechanism by which *P. haemolytica* may evade host defenses, expanding our understanding of the pathogenesis of bovine pulmonary pasteurellosis.

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