Ultrastructural characterization of apoptosis in bovine lymphocytes exposed to *Pasteurella haemolytica* leukotoxin

Yude Sun, MVSc, PhD; Kenneth D. Clinkenbeard, PhD, DVM; Charlotte L. Ownby, PhD; Laura Cudd, PhD; Cyril R. Clarke, BVSc, PhD; Sarah K. Highlander, PhD

Objective—To characterize ultrastructural changes of bovine lymphocytes exposed to *Pasteurella haemolytica* leukotoxin (LKT).

Sample Population—Partially purified LKT from a wild type *P haemolytica* A1 strain and inactive pro-LKT from an isogeneic mutant *P haemolytica* strain. Isolated bovine lymphocytes were obtained from 2 healthy calves.

Procedure—Isolated bovine lymphocytes were incubated with various concentrations of LKT and pro-LKT for 3 hours at 37 C and examined by use of transmission electron microscopy. A cytochemical Klenow DNA fragmentation assay was used to examine lymphocytes for DNA fragmentation.

Results—Lymphocytes incubated with LKT at a high concentration (1.0 toxic U/ml) had ultrastructural evidence of cytoplasmic and nuclear membrane rupture and swelling or lysis of mitochondria. Low concentrations of leukotoxin (0.1 toxic U/ml) induced DNA fragmentation in 80% of lymphocytes. Ultrastructurally, these cells had nuclear membrane blebbing, cytoplasmic vaculation, chromatin condensation, nuclear fragmentation, and membrane-bound apoptotic bodies. Incubation of lymphocytes with LKT at extremely low concentrations (0.001 toxic U/ml) or with pro-LKT did not alter their ultrastructure. Inclusion of 0.5 m*M* $ZnCl_2$ in the medium blocked leukotoxin-induced ultrastructural changes in bovine lymphocytes.

Conclusions and Clinical Relevance—Low concentrations of LKT induce apoptosis and high concentrations induce oncotic cell lysis in bovine lymphocytes. The ability of low LKT concentrations to induce apoptosis in host leukocytes may allow bacteria to escape host immune surveillance and colonize the host. (*Am J Vet Res* 2000;61:51–56)

Pneumonia caused by infection with *Pasteurella haemolytica* is a common and economically important disease in feedlot cattle.[1] During conditions that induce stress in cattle, such as shipping, viral infections, weaning, and overcrowding, *P haemolytica* proliferates in the upper respiratory tract, subsequently colonizes the lower airways, and produces various virulence factors, resulting in severe fibrinous pleuropneumonia.[2,3] **Leukotoxin (LKT)**, a protein exotoxin produced by *P haemolytica*, is considered to be the primary virulence factor in the pathogenesis of shipping fever pneumonia.[4,5] As a member of the **repeats-in-toxin (RTX)** family, LKT shares many common genetic and biochemical features with other RTX toxins.[6] Like other members of the RTX family, LKT, when present at high concentrations, is thought to form transmembrane pores in the plasma membrane of target cells, resulting in colloid-osmotic cytolysis, termed oncotic cell lysis.[7,8] In addition to oncotic cell lysis, several members of the RTX family have been found to cause apoptosis in target cells when present at low concentrations.[9-11] Killing immune and phagocytic cells by apoptosis may allow bacteria that produce these toxins to escape host immune surveillance, prolonging their survival and allowing them to colonize the host.[12-14]

Apoptosis is characterized by 2 remarkable features. One is the distinct series of morphologic changes, including nuclear chromatin condensation, cellular shrinkage, plasma membrane blebbing, and formation of membrane-bound apoptotic bodies, in cells undergoing apoptosis. The other is a specific form of DNA degradation in which the genome of the apoptotic cell is degraded at internucleosomal sites by endogenous endonucleases.[15,16] In contrast, oncotic cell lysis is typically initiated by plasma membrane permeability changes resulting in colloid-osmotic cell lysis and organelle swelling and early rupture of the cytoplasmic membrane, mitochondria, and other organelles.[8] These distinguishing features of apoptosis and oncotic cell lysis can be used to identify the specific mode of cell death.

Recently, Stevens and Czuprynski[17] reported that exposing bovine leukocytes to sublytic concentrations of *P haemolytica* LKT induces morphologic changes, detectable at the light microscopic level, consistent with apoptosis. However, DNA degradation characteristic of apoptosis was not observed, and the low resolution of light microscopy made it difficult to interpret the morphologic results. We subsequently determined that low concentrations of LKT cause DNA fragmentation characterized by internucleosomal cleavage.[18] The purpose of the study reported here was to characterize the ultrastructural changes of bovine lymphocytes after exposure to *P haemolytica* LKT and to determine whether low LKT concentrations induce ultrastructural changes consistent with apoptosis or oncotic cell lysis.

Received Nov 30, 1998.
Accepted Mar 3, 1999.
From the Department of Anatomy, Pathology and Pharmacology, College of Veterinary Medicine, Oklahoma State University, Stillwater, OK 74078 (Sun, Clinkenbeard, Ownby, Cudd, Clarke), and the Department of Microbiology and Immunology, Baylor College of Medicine, Houston, TX 77030 (Highlander).
Dr. Sun's present address is the Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, CO 80262.
Supported in part by a grant from the USDA Hatch Program OKL02249 and the Oklahoma State Agricultural Experiment Station.
The authors thank Phoebe J. Doss for technical assistance.

Materials and Methods

Isolation of bovine blood lymphocytes—Two healthy beef calves (approx 200 kg) served as blood donors for isolation of lymphocytes. Lymphocytes were isolated as described,[19] with certain modifications. Briefly, blood samples were collected by means of jugular venipuncture, and anticoagulant (sodium heparin, 15 U/ml) was added. Forty-five milliliters of heparinized blood in a 50-ml conical centrifuge tube was centrifuged at 600 × g for 30 minutes at 22 C, and plasma was aspirated down to about 10 to 15 mm above the interface between the erythrocytes and the plasma. The cells at the interface were carefully pipetted into another centrifuge tube, with as few erythrocytes as possible, and diluted (1:3, vol/vol) with **Hank's balanced salt solution (HBSS**, Ca^{2+} and Mg^{2+} free). Thirty to 35 ml of diluted cells were carefully layered on 15 ml of lymphocyte separation medium[a] and centrifuged at 500 × g for 20 minutes with the brake in the off position. After centrifugation, lymphocytes and monocytes in the white interface between the plasma and the lymphocyte separation medium were pipetted into another 50-ml conical centrifuge tube, washed twice with 30 ml of HBSS, and resuspended in RPMI-1640 containing 10% **fetal bovine serum (FBS)** at a concentration of 5×10^6 cells/ml. This final cell suspension was free of erythrocytes and contained approximately 90% lymphocytes (mean, 89.9%; range 72 to 99%) and 5% monocytes (mean, 4.5%; range 0 to 9%), as determined by means of differential cell counting. To remove monocytes, 5 ml of the cell suspension was placed in a 50-mm tissue culture-treated petri dish[b] and incubated at 37 C for 2 hours. During the incubation period, monocytes adhered to the plate surface, and 99% of nonadherent cells were lymphocytes. Greater than 95% of the cell population was capable of excluding trypan blue in all experiments.[19]

Preparation of LKT and inactive pro-LKT from concentrated culture supernatants—Concentrated culture supernatants from a wild type *P haemolytica* A1 strain SH1217 and its isogeneic strain SH1562, which contains a nonpolar insertion in the *lktC* gene, were used to obtain LKT and inactive pro-LKT preparations, respectively. The mutant SH1562 strain of *P haemolytica* produces and secrets a LKT protein that is recognized by anti-LKT monoclonal antibodies but is not acylated.[20] Concentrated culture supernatants were prepared by inoculating 1 L of RPMI-1640 medium containing 0.1*M* phosphate buffer (pH 6.8) and 1 m*M* Mg^{2+} to an optical density, at 600 nm, of 0.25 with the wild type or mutant strain of *P haemolytica* prepared by growth overnight on 5% bovine blood agar and then in 100 ml of RPMI-1640 under 5% CO_2 to the late logarithmic phase.[21] Initial cultures in RPMI-1640 were grown at 37 C with 120 oscillations/min for approximately 4 hours to an optical density of 0.9 to 1.0. All subsequent steps were conducted at 4 C. Bacteria were removed by means of centrifugation at 8,000 × g for 30 minutes, and the culture supernatant was concentrated and partially purified by means of fractional ammonium sulfate precipitation (0 to 60% saturation by addition of 361 g of solid ammonium sulfate/L). Precipitates were resuspended at a concentration of 0.5 mg of protein/ml in 20 ml of 50 m*M* sodium phosphate, 100 m*M* sodium chloride buffer, pH 7.0, dialyzed against the same buffer, and stored at –135 C. Protein concentrations of the LKT and inactive pro-LKT culture supernatant preparations were not significantly ($P > 0.05$) different.[20]

Determination of LKT activity—Activity of LKT was assayed by measuring leakage of the intracellular enzyme **lactate dehydrogenase (LDH)** from bovine lymphocytes. Cells were enumerated by use of a hemocytometer and collected by means of centrifugation at 700 × g for 15 minutes. The cell pellet was resuspended in RPMI-1640 medium, and the final concentration of bovine lymphocytes was adjusted to 4×10^6 cells/ml. Aliquots of the lymphocyte suspension (100 µl) were added to 100 µl of serial dilutions of the culture supernatants in wells on a 96-well round-bottom microtitration plate. Leakage of LDH was maximized by adding 0.1%t-octylphenoxy polyethoxyethanol (vol/vol), and background leakage was determined by exposing cells to RPMI-1640 only. Plates were incubated at 37 C for 2 hours, and exposure was terminated by means of centrifugation at 700 × g for 10 minutes. Aliquots (100 µl) of the supernatant from each well were then transferred to clean 96-well flat-bottom microtitration plates, and plates were warmed to 37 C. Activity of LDH was assessed kinetically by adding 100 µl of assay reagent,[a] held at 37 C, to all wells and measuring absorbance/min at 340 nm in a thermally controlled, kinetic microtitration plate reader[c] for 2 minutes at 37 C. Specific leakage of LDH was calculated as a percentage by use of the following equation:

$$\% \text{ specific LDH leakage} = ([A - B]/[C - B]) \times 100$$

in which A = absorbance of wells containing lymphocytes and LKT, B = absorbance of negative control (RPMI-1640) wells, and C = absorbance of positive control (t-octylphenoxy polyethoxyethanol) wells. Leukotoxin activity was then calculated in **toxic units (TU)** by graphing specific LDH leakage versus the culture supernatant dilution factor. One TU was defined as the dilution factor at which the specific LDH leakage under the assay conditions described was 50%.

Lymphocyte treatments—Isolated bovine blood lymphocytes were placed in complete RPMI-1640 medium containing 10% FBS. Effects of exposing lymphocytes to LKT were determined by incubating lymphocytes (0.5 ml, 4×10^6 cells/ml) with LKT (1.0, 0.1, and 0.001 TU/ml) or with inactive pro-LKT (diluted in the same way as LKT) for 3 hours. These concentrations of LKT were chosen on the basis of results of preliminary experiments. Cells were then pelleted by means of centrifugation at 500 × g for 10 minutes, and cell pellets were used to determine percentage of cells that were apoptotic or for transmission electron microscopy. Supernatants were assayed for LDH leakage to monitor the relationship between LKT-induced oncotic cell lysis and apoptosis in target cells; data were reported as percentage specific LDH leakage. The positive control for apoptosis consisted of incubating lymphocytes (5 ml, 4×10^6 cells/ml) with 10 µ*M* dexamethasone at 37 C for 18 hours with 5% CO_2.[18] To test the effect of Zn^{2+} on dexamethasone- or LKT-induced apoptosis, bovine lymphocytes were incubated with 10 µ*M* dexamethasone or 0.1 TU/ml of LKT in the presence of 0.5 m*M* Zn^{2+} and then examined for percentage of apoptotic cells or the ultrastructural changes.

Quantification of percentage of apoptotic lymphocytes—Percentage of bovine lymphocytes with apoptosis was determined by use of a cytochemical Klenow DNA fragmentation assay[d] that uses a DNA fragment end-labeling technique. Lymphocytes that had been incubated with LKT, inactive pro-LKT, or dexamethasone were fixed and immobilized on glass slides, using a cytocentrifuge.[e] Slides were stained according to the manufacturer's instructions, and percentage of apoptotic cells was determined by counting 100 cells.

Transmission electron microscopy—Samples were prepared for electron microscopy using a modification of a described protocol.[22] Briefly, aliquots (0.5 ml) of control and treated cells (4×10^6 cells/ml) were pelleted by means of centrifugation at 500 × g for 1 minute and washed twice with 0.1 *M* cacodylate buffer (pH 7.4). Cells were fixed in 1.6% glutaraldehyde in 0.2 *M* cacodylate buffer (pH 7.4), and fixed cells were washed in 0.1 *M* cacodylate buffer and postfixed in 1%

osmium tetroxide in 0.2 *M* cacodylate buffer for 2 hours. Cells were washed and then dehydrated by passing them through a graded series of ethanol solutions (50, 70, 90, 95, and 100%). They were then transferred to propylene oxide for 1 hour and, finally, embedded in resin.[f] Thin sections (70 to 90 nm) of selected areas of the resin blocks were cut with an ultramicrotome,[g] using a diamond knife.[h] Sections were mounted on copper grids (200 mesh), stained with aqueous uranyl acetate and lead citrate, and examined with a transmission electron microscope[i] at an accelerating voltage of 80 kV.

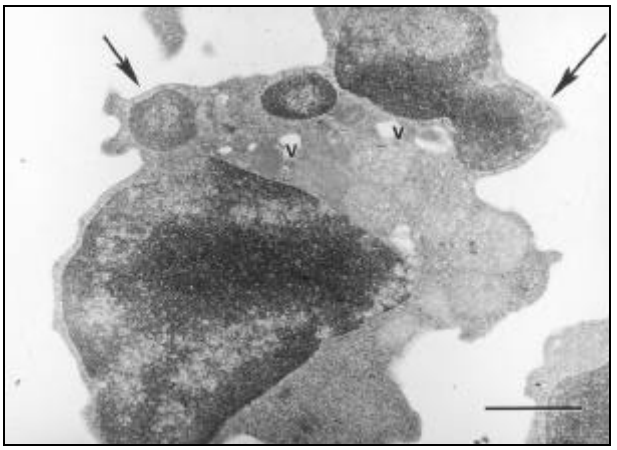

Figure 1—Transmission electron micrograph of bovine lymphocytes incubated with 10 μ*M* dexamethasone for 18 hours at 37 C. Cytoplasmic vacuolation (v), chromatin condensation and margination, nuclear lobulation, and membrane-bound apoptotic bodies (arrows) were evident. Bar = 1 μm.

Results

As determined by a cytochemical Klenow DNA fragmentation assay, 82% of bovine lymphocytes incubated with dexamethasone and 80% of lymphocytes incubated with LKT at a concentration of 0.1 TU/ml underwent DNA fragmentation characteristic of apoptosis, whereas only 5% of lymphocytes incubated with pro-LKT at a similar dilution underwent apoptosis. Only 4% of lymphocytes incubated with LKT at a concentration of 0.1 TU/ml and 0.5 mM $ZnCl_2$ had evidence of apoptosis.

Incubation of lymphocytes with LKT at a concentration of 1.0 TU/ml resulted in 55% specific LDH leakage, and incubation with LKT at a concentration of 0.1 TU/ml resulted in only 5% specific LDH leakage. Incubation of lymphocytes with LKT at a concentration of 0.001 TU/ml or with any of the dilutions of inactive pro-LKT did not induce specific LDH leakage.

Bovine lymphocytes incubated with 10 μ*M* dexamethasone for 18 hours at 37 C had typical ultrastructural features of apoptosis, including cytoplasmic vacuolation, chromatin condensation and margination, nuclear

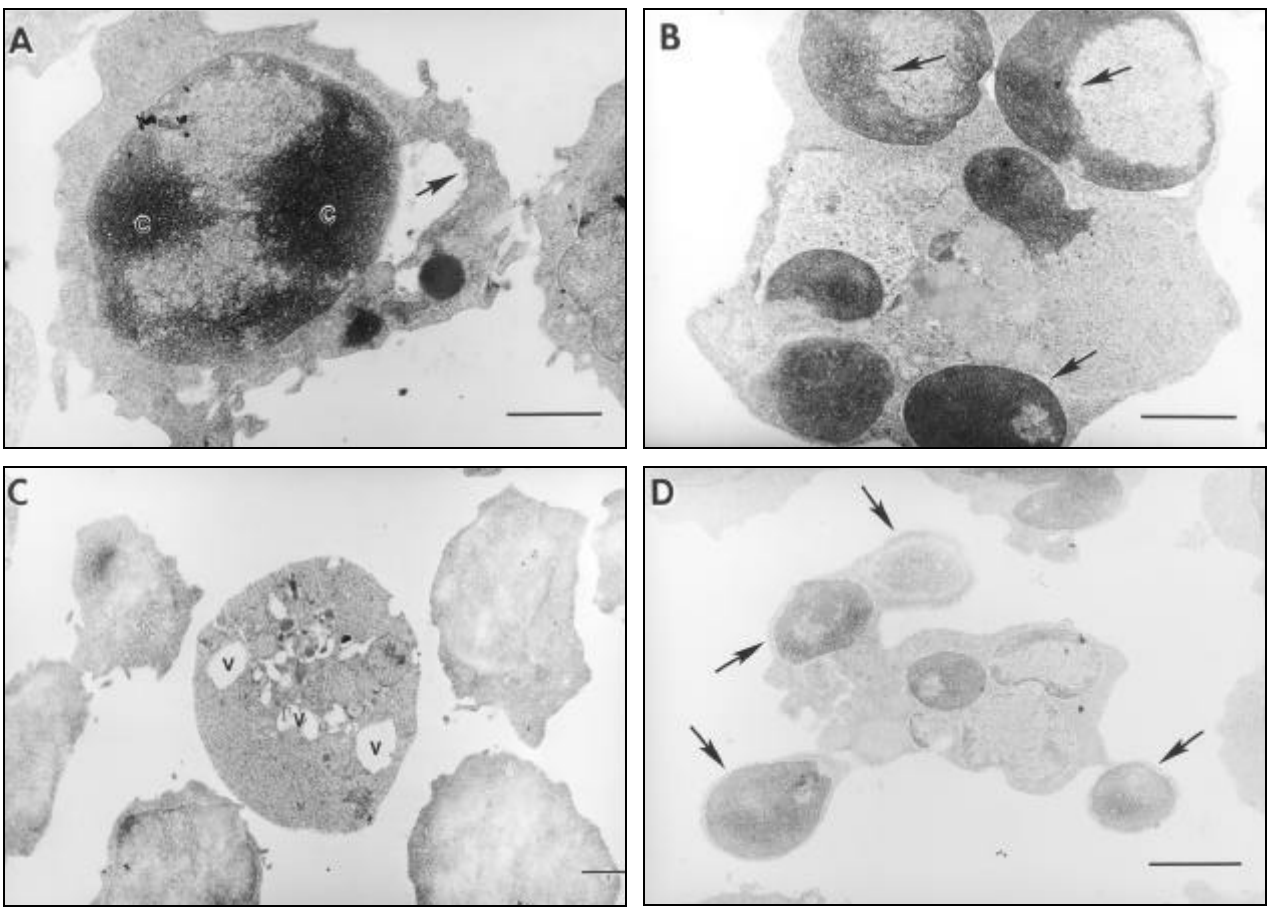

Figure 2—Transmission electron micrographs of bovine lymphocytes incubated with *Pasteurella haemolytica* leukotoxin (0.1 toxic U/ml) for 3 hours. A—Notice the chromatin condensation and margination (c) and nuclear membrane blebbing (arrow). B—Notice the nuclear fragmentation and chromatin condensation in a horseshoe shape (arrows). C—Notice the variably sized cytoplasmic vacuoles (v). D—Notice the membrane-bound apoptotic bodies (arrows). Bars = 1 μm.

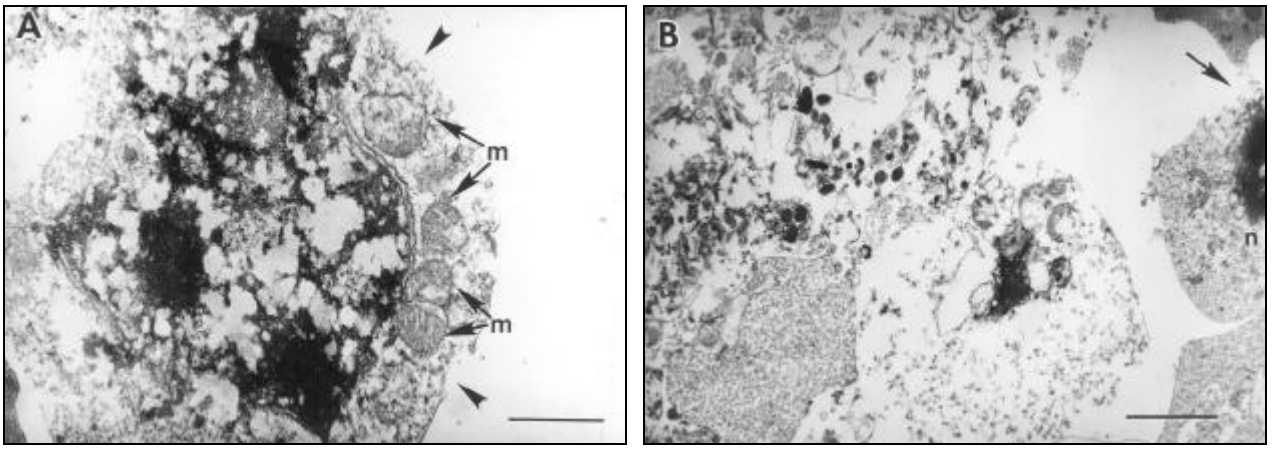

Figure 3—Transmission electron micrographs of bovine lymphocytes incubated with *P haemolytica* leukotoxin (1.0 toxic U/ml) for 3 hours. A—Notice the rupture of the cytoplasmic membrane (arrowheads) and damaged mitochondria (m). B—Notice the condensation of nuclear chromatin (n) and rupture of the nuclear envelope (arrow). Bar = 1 μm.

lobulation, and membrane-bound apoptotic bodies (**Fig 1**), whereas the ultrastructural appearance of bovine lymphocytes incubated in RPMI-1640 for 18 hours was normal. These cells had scant cytoplasm and a large, slightly indented nucleus; the nuclear chromatin had a condensed heterochromatin-euchromatin pattern.

Incubation of lymphocytes with a low concentration of *P haemolytica* LKT (0.1 TU/ml) for 3 hours induced marked alterations in the cell ultrastructure, including irregularities in cell shape and nuclear and cytoplasmic abnormalities (**Fig 2**). Nuclear membrane blebbing was common. Nuclei with irregular nuclear shapes were either fragmented, condensed, or polylobated. Condensed chromatin in the shape of a horseshoe was frequently observed at the periphery of the nucleus, and cytoplasmic vacuoles and apoptotic bodies of various sizes and shapes were observed. Ultrastructural changes in these cells were similar to those seen in bovine lymphocytes incubated with dexamethasone. Addition of 0.5 m*M* Zn^{2+} to the culture medium apparently blocked the effects of LKT (0.1 TU/ml) and dexamethasone, as lymphocytes incubated with 0.5 m*M* Zn^{2+}, in addition to LKT or dexamethasone, did not have any ultrastructural abnormalities.

Incubation of lymphocytes with LKT at a concentration of 0.001 TU/ml or with various concentrations of inactive pro-LKT did not alter their ultrastructural appearance, and ultrastructural appearance of these cells was similar to that of bovine lymphocytes incubated in RPMI-1640. Incubation of bovine lymphocytes with LKT at the highest concentration (1.0 TU/ml) resulted in rupture of nuclear, mitochondrial, and plasma membranes (**Fig 3**). In some cells, mitochondria were swollen with ruptured cristae, and the nuclear envelope and plasma membrane were ruptured. Some cells were completely lysed, with only a remnant of membrane present; however, other cells with ruptured cytoplasmic membranes contained some intact organelles and discernable nuclei.

Discussion

Apoptosis and oncotic cell lysis are 2 distinct mechanisms of cell death, each of which has its own typical morphologic and biochemical features.[23] Criteria used for classifying cells as apoptotic generally include demonstration of typical ultrastructural abnormalities by use of electron microscopy and identification of a DNA ladder pattern of multiple 200 base pair fragments by use of agarose gel electrophoresis.[9,23] In the present study, we used transmission electron microscopy to examine the morphology of the treated cells, but rather than using gel electrophoresis to detect DNA fragmentation, we used a cytochemical Klenow DNA fragmentation assay. This assay detects apoptotic cells on the basis of 3'-OH end labeling with biotinylated nucleotides catalyzed by the Klenow enzyme. The biotin-labeled DNA is then detected by addition of streptavidin-horseradish peroxidase conjugate.

In this study, we found that bovine lymphocytes exposed to *P haemolytica* LKT at a concentration of 0.1 TU/ml had ultrastructural abnormalities typical of apoptosis, including chromatin condensation and margination, nuclear fragmentation, cytoplasmic vacuoles, and membrane-bound apoptotic bodies.[9,10,16,23-30] Apoptotic bodies were surrounded by an intact membrane, indicating that there had not been any spillage of intracellular contents. Therefore, conventional methods for determining cell death, such as ^{51}Cr release, LDH leakage assays, and trypan blue exclusion test, may underestimate the proportion of dead cells,[23] because these methods depend on an increase in plasma membrane permeability associated with cell death.

Leukotoxin-induced oncotic cell lysis of bovine leukocytes has been studied by measuring ^{51}Cr release or intracellular enzyme leakage or by performing trypan blue exclusion assays or scanning electron microscopy.[31] These methods evaluate integrity of the cytoplasmic membranes or demonstrate the surface appearance of the target cells and can be used to estimate the severity of damage in a population of cells. However, intracellular ultrastructural changes of individual cells cannot be determined. In the present study, we used transmission electron microscopy to examine ultrastructural changes in individual cells. Chromatin condensation, which is usually used as a criterion for identification of apoptosis, was observed in cells undergoing oncotic lysis. However,

on electron micrographs, the chromatin condensation associated with oncotic cell lysis was easily distinguished from that associated with apoptosis, because with cells undergoing apoptosis, the nuclear membrane was intact.

The components of the culture supernatants used in this study were similar except that the mutant *P haemolytica* strain only produced an inactive non-acylated pro-LKT.[20] Treatment of bovine lymphocytes with various concentrations of inactive pro-LKT did not alter their ultrastructural morphology, indicating that the active component of the wild type *P haemolytica* culture supernatants was LKT. This also implies that post-translational modification of an RTX is required for the toxin to induce apoptosis or oncotic cell lysis. Results of the present study indicated that a high concentration of LKT caused oncotic cell lysis and a low concentration caused apoptosis. Extremely low concentrations of LKT did not cause cell death.

The effects of zinc on the apoptosis pathway have been extensively studied.[24,25] In a previous study,[18] zinc effectively blocked dexamethasone- and LKT-induced DNA fragmentation, suggesting that zinc may act as a potent endonuclease inhibitor. However, DNA fragmentation is a relatively late event in the apoptosis pathway, and therefore, blocking DNA fragmentation alone would not be sufficient to prevent the ultrastructural abnormalities typical of bovine lymphocytes incubated with dexamethasone or LKT. Recently, it has been reported that zinc is also a potent caspase inhibitor, and caspase activation is an early event in the apoptosis pathway.[25,32] It is likely that dexamethasone and LKT induce apoptosis by activating caspases,[33,34] and our finding that zinc blocks dexamethasone- and LKT-induced morphologic changes typical of apoptosis supports the suggestion that zinc inhibits caspase activation.

Leukotoxin-induced apoptosis of lymphocytes could explain the LKT-mediated inhibition of mitogen-induced blastogenesis of bovine or ovine lymphocytes in vivo.[35-37] Induction of apoptosis in cells directly involved in the immune response, as shown in the case of LKT from *P haemolytica*, may enhance initiation and extension of bacterial infections.[38]

[a]LDH-L 50, Sigma Chemical Co, St Louis, Mo.
[b]Nalge Nunc International, Milwaukee, Wis.
[c]Thermomax, Molecular Devices Palo Alto, Calif.
[d]Oncogene Research Products, Cambridge, Mass.
[e]Shandon Lipshaw, Pittsburgh, Pa.
[f]Polysciences, Warrington, Pa.
[g]Du Pont Co, Diagnostic and BioResearch Systems, Wilmington, Del.
[h]Diatome US, Fort Washington, Pa.
[i]JEOL LTD, Tokyo, Japan.

References

1. Yates WDG. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Can J Comp Med* 1982;46:225–263.

2. Confer AW, Panciera RJ, Mosier DA. Bovine pneumonic pasteurellosis: immunity to *Pasteurella haemolytica*. *J Am Vet Med Assoc* 1988;193:1308–1316.

3. Whiteley LO, Maheswaran SK, Weiss DJ, et al. *Pasteurella haemolytica* A1 and bovine respiratory disease: pathogenesis. *J Vet Intern Med* 19926:11–22.

4. Petras SF, Chidambaram M, Illyes EF, et al. Antigenic and virulence properties of *Pasteurella haemolytica* leukotoxin mutants. *Infect Immun* 1995;63:1033–1039.

5. Tatum FM, Briggs RE, Sreevatsan SS, et al. Construction of an isogeneic leukotoxin deletion mutant of *Pasteurella haemolytica* serotype 1: characterization and virulence. *Microb Pathog* 1998;24:37–46.

6. Welch RA. Pore-forming cytolysins of gram-negative bacteria. *Mol Microbiol* 1991; 5:521–528.

7. Clinkenbeard KD, Mosier DA, Confer AW. Transmembrane pore size and role of cell swelling in cytotoxicity caused by *Pasteurella haemolytica* leukotoxin. *Infect Immun* 1989;57:420–425.

8. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* 1995;146:3–15.

9. Mangan DF, Taichman NS, Lally ED, et al. Lethal effects of *Actinobacillus actinomycetemcomitans* leukotoxin on human T lymphocytes. *Infect Immun* 1991;59:3267–3272.

10. Kato S, Muro M, Akifusa S, et al. Evidence for apoptosis of murine macrophages by *Actinobacillus actinomycetemcomitans* infection. *Infect Immun* 1995;63:3914–3919.

11. Korostoff J, Wang JF, Kieba I, et al. *Actinobacillus actinomycetemcomitans* leukotoxin induces apoptosis in HL-60 cells. *Infect Immun* 1998;66:4474–4483.

12. Chen Y, Zychlinsky A. Apoptosis induced by bacterial pathogens. *Microb Pathog* 1994;17:203–212.

13. Finlay BB, Cossart P. Exploitation of mammalian host cell functions by bacterial pathogens. *Science* 1997;276:718–725.

14. Zychlinsky A, Sansonetti P. Perspectives series: host/pathogen interactions. Apoptosis in bacterial pathogenesis. *J Clin Invest* 1997;100:493–495.

15. Wyllie AH, Morris RG, Smith AL, et al. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J Pathol* 1984;142:67–77.

16. Machaca K, Compton MM. Analysis of thymic lymphocyte apoptosis using in vitro techniques. *Dev Comp Immunol* 1993;17: 263–276.

17. Stevens PK, Czuprynski CJ. *Pasteurella haemolytica* leukotoxin induces bovine leukocytes to undergo morphologic changes consistent with apoptosis in vitro. *Infect Immun* 1996;64:2687–2694.

18. Sun Y, Clinkenbeard KD, Clarke C, et al. *Pasteurella haemolytica* leukotoxin induced apoptosis of bovine lymphocytes involves DNA fragmentation. *Vet Microbiol* 1999;65:153–166.

19. Reeves JH, Renslaw HW. Surface membrane markers on bovine peripheral blood lymphocytes. *Am J Vet Res* 1987;39:917–923.

20. Fedorova ND, Highlander SK. Generation of targeted nonpolar gene insertions and operon fusions in *Pasteurella haemolytica* and creation of a strain that produces and secretes inactive leukotoxin. *Infect Immun* 1997;65:2593–2598.

21. Sun Y, Clinkenbeard KD. Serum-free culture of *Pasteurella haemolytica* optimized for leukotoxin production. *Am J Vet Res* 1988;59:851–855.

22. Anderson SG, Ownby CL. Systemic hemorrhage induced by proteinase H from *Crotalus adamannteus* (eastern diamondback rattlesnake) venom. *Toxicon* 1997;35:1301–1313.

23. Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251–306.

24. Solary E, Dubrez L, Eymin B. The role of apoptosis in the pathogenesis and treatment of diseases. *Eur Respir J* 1996;9:1293–1305.

25. Perry DK, Smyth MJ, Stennicke HR, et al. Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis. *J Biol Chem* 1997;272:18530–18533.

26. Allen PD, Bustin SA, Newland AC. The role of apoptosis (programmed cell death) in haemopoiesis and the immune system. *Blood Rev* 1993;7:63–73.

27. Fady C, Garddie A, Jacoby F, et al. Atypical apoptotic cell death induced in L929 by exposure to tumor necrosis factor. *J Interferon Cytokine Res* 1995;15:71–80.

28. Cohen JJ. Apoptosis and its regulation. In: Gupta S, Cohn JJ, eds. *Mechanisms of lymphocyte activation and immune regulation VI*. New York: Plenum Press, 1996;11–21.

29. Lieberthal W, Levine JS. Mechanisms of apoptosis and its potential role in renal tubular epithelial cell injury. *Am J Physiol* 1996;271:477–488.

30. Gueirard P, Druilhe A, Pretolani M, et al. Role of adenylate cyclase-hemolysin in alveolar macrophage apoptosis during *Bordetella pertussis* infection in vivo. *Infect Immun* 1998;66:1718–1725.

31. Clinkenbeard, KD, Mosier DA, Timko AL, et al. Effects of

Pasteurella haemolytica leukotoxin on cultured bovine lymphoma cells. *Am J Vet Res* 1989;50:271–275.

32. Stennicke HR, Salvesen GS. Biochemical characteristics of caspases–3, –6, –7, and –8. *J Biol Chem* 1997;272:25719–25723.

33. Wang JF, Kieba IR, Korostoff J, et al. Molecular and biochemical mechanisms of *Pasteurella haemolytica* leukotoxin-induced cell death. *Microb Pathog* 1998;25:317–331.

34. Ivanov VN, Nikolic-Zugic J. Biochemical and kinetic characterization of the glucocorticoid-induced apoptosis of immature CD4+CD8+ thymocytes. *Int Immunol* 1998;10:1807–1817.

35. Majury AL, Shewen PE. The effect of *Pasteurella haemolytica* A1 leukotoxic culture supernate on the in vitro proliferative response of bovine lymphocytes. *Vet Immunol Immunopathol* 1991;29:41–56.

36. Majury AL, Shewen PE. Preliminary investigation of the mechanism of inhibition of bovine lymphocyte proliferation by *Pasteurella haemolytica* A1 leukotoxin. *Vet Immunol Immunopathol* 1991;29:57–68.

37. Sharma R, Woldehiwet Z. Lymphocyte subpopulations in peripheral blood of lambs experimentally infected with *Pasteurella haemolytica*. *Vet Microbiol* 1991;27:159–168.

38. Finlay BB, Falkow S. Common themes in microbial pathogenicity revisited. *Mol Biol Rev* 1997;61:136–169.