INFECTION AND IMMUNITY, Feb. 1989, p. 420–425
0019-9567/89/020420-06$02.00/0

Vol. 57, No. 2

Transmembrane Pore Size and Role of Cell Swelling in Cytotoxicity Caused by *Pasteurella haemolytica* Leukotoxin†

KENNETH D. CLINKENBEARD,* DEREK A. MOSIER, AND ANTHONY W. CONFER

Department of Veterinary Pathology, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078

Received 12 July 1988/Accepted 24 October 1988

Pasteurella haemolytica **A1 leukotoxin causes rapid (5 to 15 min) leakage of intracellular K^+ and cell swelling and slower (15 to 60 min), Ca^{2+}-dependent formation of large plasma membrane defects (≈100 nm) and leakage of lactate dehydrogenase from bovine lymphoma cells (BL3 cells) (K. D. Clinkenbeard, D. A. Mosier, A. L. Timko, and A. W. Confer, Am. J. Vet. Res., in press). Incubation of BL3 cells in medium made hypertonic by inclusion of 75 mM sucrose blocked leukotoxin-induced cell swelling, formation of large plasma membrane defects, and leakage of lactate dehydrogenase but did not block leukotoxin-induced leakage of intracellular K^+. Carbohydrates with molecular weights less than that of sucrose, e.g., mannitol, did not block leukotoxin-induced cell swelling of BL3 cells. Increasing the concentration of mannitol to twice that of sucrose still resulted in no protective effect. Assuming that leukotoxin acts as a transmembrane molecular sieve, then the functional transmembrane pore size formed by leukotoxin in BL3 cells is slightly less than the size of sucrose, i.e., 0.9 nm. Exposure of BL3 cells to leukotoxin for 15 or 45 min followed by the addition of hypertonic sucrose to the incubation medium reversed leukotoxin-induced cell swelling and prevented further leakage of lactate dehydrogenase. Leukotoxin-induced leakage of lactate dehydrogenase required both cell swelling and Ca^{2+}-dependent processes. The Ca^{2+}-dependent steps can occur before or concurrent with cell swelling.**

Pasteurella haemolytica A1, the most common bacterium isolated from the lungs of cattle with pneumonic pasteurellosis (9), produces an exotoxin (leukotoxin) that damages ruminant leukocytes (1, 2, 15). Leukotoxin, which is composed of 105,000-molecular-weight subunits, has an apparent molecular weight of >300,000 (8). A DNA fragment coding for *P. haemolytica* leukotoxin has been isolated and shares extensive homology with the gene coding for *Escherichia coli* α-hemolysin (11, 16). Exposure of bovine leukocytes to leukotoxin results in rapid leakage of intracellular K^+ (<5 min) and cell swelling (<15 min). A slower Ca^{2+}-dependent formation of large (≈100-nm) plasma membrane defects and leakage of large cytoplasmic components into the media, e.g., lactate dehydrogenase (LDH), occurs between 15 and 60 min of leukotoxin exposure (K. D. Clinkenbeard, D. A. Mosier, A. L. Timko, and A. W. Confer, Am. J. Vet. Res., in press).

It appears that *P. haemolytica* leukotoxin injures its target cells by a mechanism similar to that of several other bacterial cytolysins, i.e., insertion of the toxin into the plasma membrane with resulting formation of transmembrane pores (6). These pores allow intracellular K^+ to leak from the cell, but larger cytoplasmic components such as proteins are initially retained within the cell. This toxin-induced molecular sieving results in the intracellular osmotic pressure becoming greater than that in the medium, and the cells swell. After swelling, the cells begin to leak larger cytoplasmic components (3).

Inclusion of certain carbohydrates in the incubation medium prevents cell swelling caused by pore-forming cytolysins (12). The ability of these agents, termed osmotic stabilizing or protecting agents, to prevent cell swelling is proportional to their molecular weight and concentration in the medium. It has been proposed that osmotic stabilizing agents prevent toxin-induced cell swelling by causing the medium to be hypertonic compared with the cytoplasm. For this to occur, the molecular weight of the osmotic stabilizing agent must be high enough that it cannot pass through the toxin-formed pores into the cytoplasm, and the concentration of the agent in the medium must be sufficient to counterbalance the toxin-induced increase in intracellular osmotic pressure.

Based on the assumption that pore-forming cytolysins act as molecular sieves, osmotic stabilizing agents have been used to estimate the size of toxin-formed transmembrane pores (4). A series of carbohydrates of differing molecular weights were used as osmotic stabilizing agents with *P. haemolytica* leukotoxin and cultured bovine lymphoma cells, and the effectiveness of carbohydrates of various molecular weights in preventing toxin-induced cell swelling was used to estimate the size of the transmembrane pores formed by *P. haemolytica* leukotoxin. Osmotic stabilizing agents were also used to examine the role of cell swelling in leukotoxin-induced plasma membrane damage and its relationship to Ca^{2+}-dependent membrane damage.

MATERIALS AND METHODS

Cultivation techniques. Lyophilized cultures of *P. haemolytica* A1 were suspended in distilled water, plated onto enriched brain heart infusion agar, and grown at 37°C under 5% CO_2 for 18 h. Isolated colonies were transferred to 50 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) in a 250-ml Erlenmeyer flask and grown at 37°C with rotatory shaking at 80 oscillations per min for 4 h. These

* Corresponding author.

† Journal article 88-018 from the College of Veterinary Medicine and the Agricultural Experiment Station, Oklahoma State University.

cultures were used to inoculate media for the production of leukotoxin.

Leukotoxin production. Cultures of *P. haemolytica* prepared as described above were collected by centrifugation (Sorvall rotor SS34; 12,000 × *g* for 30 min at 25°C) and suspended in 10 ml of 3.5% bovine serum albumin in RPMI 1640 medium (Sigma Chemical Co., St. Louis, Mo.) at pH 7.2. The suspended bacteria were used to inoculate 100 to 400 ml of RPMI 1640–3.5% bovine serum albumin in Erlenmeyer flasks, which were incubated at 37°C for 1 h and then centrifuged as described above. The supernatant (leukotoxin) was filtered through a 0.22-μm filter (Nalgene Filtunit TYP S CN; Nalge Company, Rochester, N.Y.) and stored at −70°C. Heat-denatured leukotoxin was prepared by incubating leukotoxin at 60°C for 10 min.

Cultivation of bovine lymphoma cells. Bovine lymphoma cells (BL3 cells from G. Theilen, University of California, Davis) were grown in suspension culture in 50% Leibovitz L-15–50% Eagle minimal essential medium with 20% fetal bovine serum to which were added L-glutamine (2 mmol $liter^{-1}$), gentamicin (50 mg $liter^{-1}$) and $NaHCO_3$ (2.2 g $liter^{-1}$) at 37°C with 5% CO_2 (GIBCO Laboratories, Grand Island, N.Y.). Cells used for leukotoxin assays were split 1:1 in the above media and grown for 1 day before use. The cell concentration was determined by manual cell counts (Unopette System 5856; Becton-Dickinson Vacutainer Systems, Rutherford, N.J.). The cells were harvested by centrifugation (IEC Centrac rotor 216; 700 × *g* for 10 min at 25°C), the supernatant was discarded, and the pellet was suspended at the desired cell concentration for use.

Characterization of leukotoxin. Three leukotoxin preparations were used in these experiments. The leukotoxin preparations had half-lives of inactivation at 60°C of 1.3 min for K^+ leakage and 1.5 min for LDH leakage from BL3 cells. Leukotoxin preparations caused leakage of K^+ and LDH from isolated bovine neutrophils and lymphocytes and BL3 cells but not from bovine erythrocytes or cultured Madin-Darby bovine kidney cells. Antibodies to purified leukotoxin (14) neutralized ≥90% of the leukotoxin activity of these preparations as assessed by LDH leakage from BL3 cells.

Potassium release assay. BL3 cells (5.6×10^7 cells per ml) in 0.5 ml of 10 mM Tris–140 mM NaCl (pH 7.3) with or without 75 mM sucrose were incubated at 25°C for 20 min with heat-denatured leukotoxin or native leukotoxin (240 TU). The cells were removed by centrifugation (Triac microhemocrit rotor; 11,700 × *g* for 3 min; Clay-Adams, Rutherford, N.J.) in microhematocrit tubes, and the K^+ in the supernatant was measured by using an ion-specific electrode (ACA11-60; Du Pont Co., Wilmington, Del.).

PCV. Assay mixtures were incubated with oscillatory shaking (50 oscillations per min) for the time periods indicated. Samples of the assay mixtures were transferred to microhematocrit tubes and centrifuged as described above. The packed cell volume (PCV) was derived by dividing the height of the column of packed cells by the height of the column of packed cells plus the fluid column multiplied by 100 and expressed as the percentage of the total volume.

LDH activity release assay. Assay mixtures were incubated with oscillatory shaking (50 oscillations per min) for the time periods indicated. Samples were then centrifuged (Surespin centrifuge; Helena Laboratories, Beaumont, Tex.; 5,800 × *g* for 2 min), and LDH activity in the supernatant was measured spectrophotometrically (LDL50; Sigma) (Gilford System 5 Clinical Chemistry Analyzer; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 30°C.

TU. One toxic unit (TU) is the amount of leukotoxin that caused ≥50% leakage of the total LDH activity from 5×10^5 BL3 cells in 0.25 ml of RPMI after 120 min of incubation at 37°C. Leukotoxin preparations typically contained 8,000 TU/ml.

TABLE 1. Osmotic protection of BL3 cells from leukotoxin-induced cell swelling and LDH leakage[a]

Carbohydrate (mM)	Molecular weight	Change in PCV[b] (%)	LDH leakage[c] (mU per assay)
None		30.7 ± 0.6	325.5 ± 45.8
Glycerol (75)	92.1	31.3 ± 1.5	315.3 ± 68.2
Mannitol (75)	182.2	31.3 ± 0.6	175.7 ± 12.8
Sedoheptulose (75)	210.2	30.0 ± 1.0	171.9 ± 8.4
N-Acetylmannosamine (75)	239.2	24.3 ± 1.2	51.4 ± 21.7
Sucrose (75)	342.3	5.3 ± 0.6	65.8 ± 12.8
Inulin (25)	≈5,000	−2.7 ± 0.6	46.7 ± 9.7

[a] BL3 cells (10^8) in 0.5 ml of 10 mM Tris–140 mM NaCl (pH 7.3) containing 1 mM $CaCl_2$ and carbohydrate as indicated were incubated with native or heat-denatured leukotoxin (100 TU) at 37°C.

[b] LKT-induced PCV after 30 min of incubation minus control PCV (mean ± standard deviation for three samples). The initial PCV was 19%.

[c] LKT-induced LDH leakage (expressed as mU per 10-μl assay out of a 500-μl total assay volume) after 120 min of incubation minus LDH leakage from control cells incubated with heat-denatured LKT in the same incubation medium (mean ± standard deviation, assay volume for three samples). Triton X-100 released 617 mU per assay.

Scanning electron microscopy. BL3 cells (5×10^7) in 0.5 ml of RPMI 1640 medium with and without 75 mM sucrose were incubated with or without leukotoxin (100 TU) at 37°C for 30 min. After incubation, cells were fixed in 2.5% glutaraldehyde, washed in cacodylate buffer, attached to 1% polylysine-coated cover slips, and dehydrated by sequential washes in increasing strengths of ethanol (50 to 100%). Cover slips were critical point dried, mounted on aluminum stubs, coated with gold-palladium, and examined with a scanning electron microscope (JSM 35-U; JEOL Ltd., Tokyo, Japan).

Reproducibility of experimental data. The data reported are means for three or more samples or are representative data for experiments that were conducted two or more times with essentially identical results.

RESULTS

Exposure of bovine lymphoma cells (BL3 cells) to *P. haemolytica* leukotoxin resulted in rapid cell swelling as measured by increased PCV (Table 1). The protective effects of osmotic stabilizing agents on leukotoxin-induced cell swelling were tested by using carbohydrates of molecular weights ranging between 92.1 and 5,000. Leukotoxin-induced cell swelling was decreased for media containing carbohydrates with molecular weights of >239.2 (Table 1). Although inclusion of 75 mM sucrose blocked leukotoxin-induced cell swelling, it did not block leukotoxin-induced leakage of intracellular K^+ (Table 2). In addition to K^+ leakage and cell swelling, leukotoxin exposure of BL3 cells caused leakage of the cytoplasmic enzyme LDH into the incubation medium (Table 1). Inclusion of osmotic stabilizing agents with molecular weights of ≥182.2 reduced the leukotoxin-induced leakage of LDH activity.

The concentration dependence of sucrose protection of BL3 cells from leukotoxin-induced cell swelling and LDH leakage is shown in Fig. 1. Increasing sucrose concentrations caused a steady decrease in cell swelling and LDH leakage. Maximal protective effects were reached at sucrose

TABLE 2. Sucrose prevents leukotoxin (LKT)-induced cell swelling but not K^+ leakage from BL3 cells

Incubation conditions	K^+ leakage (μEq)[a]	PCV (%)[b]
No sucrose		
Heat-denatured LKT	2.88 ± 0.00	10.8 ± 0.7
Native LKT	20.43 ± 0.27	19.7 ± 0.5
Sucrose (75 mM)		
Heat-denatured LKT	2.97 ± 0.03	9.3 ± 0.5
Native LKT	20.13 ± 0.36	10.0 ± 0.6

[a] Mean ± standard deviation for three samples; 21.00 μEq of K^+ was released by 0.1% Triton X-100.
[b] Mean ± standard deviation for six samples.

concentrations of 54 mM for cell swelling and 38 mM for LDH leakage. In contrast, increasing the concentration of mannitol in the incubation medium did not protect BL3 cells from leukotoxin-induced cell swelling or LDH leakage (Fig. 2), even when the concentration of mannitol was twice that of sucrose. Increasing sucrose or mannitol concentrations did cause control cells to shrink during the 30-min incubation period (maximal decrease in PCV was 2%), but mannitol or sucrose concentrations of 150 or 75 mM, respectively, had no effect on background or total cellular LDH activity.

The ability of sucrose to protect BL3 cells preexposed to leukotoxin for 15 or 45 min was examined to determine whether the osmotic protection was lost under these conditions. The addition of sucrose to the incubation medium at a final concentration of 75 mM after either 15 or 45 min of leukotoxin exposure reversed the leukotoxin-induced cell swelling and stopped further leukotoxin-induced leakage of LDH (Fig. 3). This protective effect was observed after 45 min of leukotoxin exposure when ≈50% of the cellular LDH had been leaked.

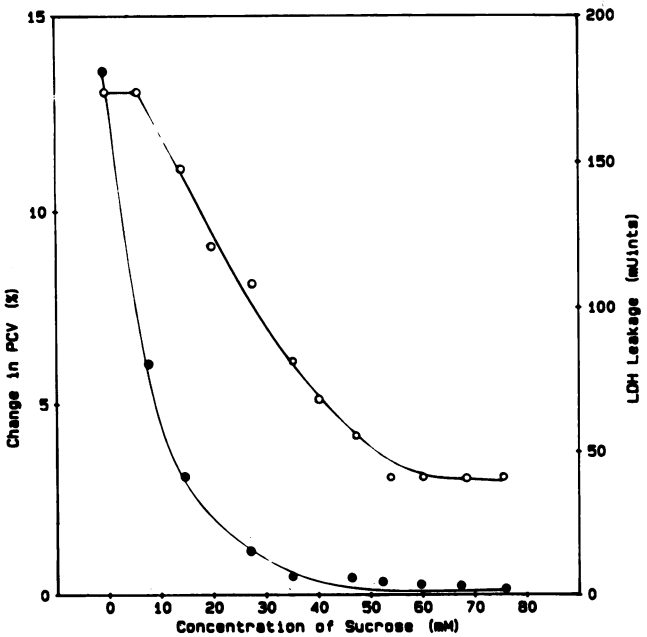

FIG. 1. Osmotic protection by sucrose of BL3 cells exposed to leukotoxin. BL3 cells (2.8×10^7) suspended in 0.25 ml of RPMI containing 0 to 75 mM sucrose were exposed to leukotoxin (40 TU). PCV (○) and LDH activity (●) were measured at 30 and 120 min, respectively.

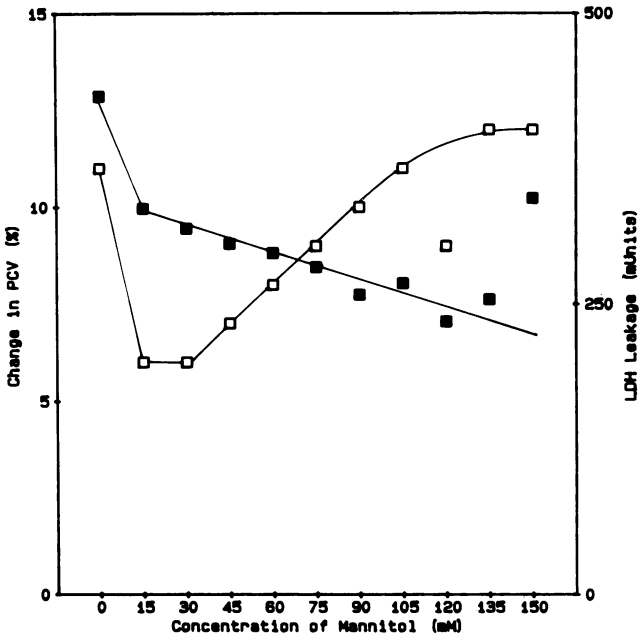

FIG. 2. Lack of osmotic protection by mannitol of BL3 cells exposed to leukotoxin. Experiment conditions were as described in the legend to Fig. 1, except that cells were suspended in RPMI containing 0 to 150 mM mannitol and exposed to leukotoxin (100 TU). Symbols: □, PCV; ■, LDH activity.

Exposure of BL3 cells to leukotoxin in medium containing Ca^{2+} resulted in the formation of large plasma membrane defects as observed by scanning electron microscopy (Fig. 4). After 30 min of exposure of BL3 cells to leukotoxin, the cells were larger (Fig. 4c) than BL3 cells not exposed to leukotoxin (Fig. 4a), and many had large plasma membrane defects (Fig. 4c). In contrast, BL3 cells that were exposed to leukotoxin for 30 min in incubation medium containing 75 mM sucrose developed a finely porous appearance to the plasma membrane and some large membrane blebs (Fig. 4d), but these cells did not become enlarged or develop large plasma membrane defects. Observation by electron microscopy of cell swelling and formation of large plasma membrane defects caused by exposure to leukotoxin also correlated with leakage of LDH activity by these cells. Cells not exposed to leukotoxin (Fig. 4a and b) and those exposed tc leukotoxin in the presence of 75 mM sucrose (Fig. 4d) leaked ≈10% of their total cellular LDH activity, whereas the BL3 cells exposed to leukotoxin in the absence of added sucrose (Fig. 4c) leaked ≈90% of their total cellular LDH activity.

The relationship between leukotoxin-induced cell swelling and Ca^{2+}-dependent LDH leakage was examined by exposing BL3 cells to leukotoxin in sucrose-containing medium to prevent cell swelling and then suspending the exposed cells in medium lacking sucrose to allow cell swelling. Initially, BL3 cells were incubated with leukotoxin in medium containing 75 mM sucrose with either 1 mM Ca^{2+} or 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] for 30 min, after which the cells were suspended in medium lacking sucrose but containing either 1 mM Ca^{2+} or 5 mM EGTA. Initial incubation in medium containing sucrose and Ca^{2+} resulted in rapid leakage of LDH from the cells after suspension in medium lacking sucrose, even when the suspension medium did not

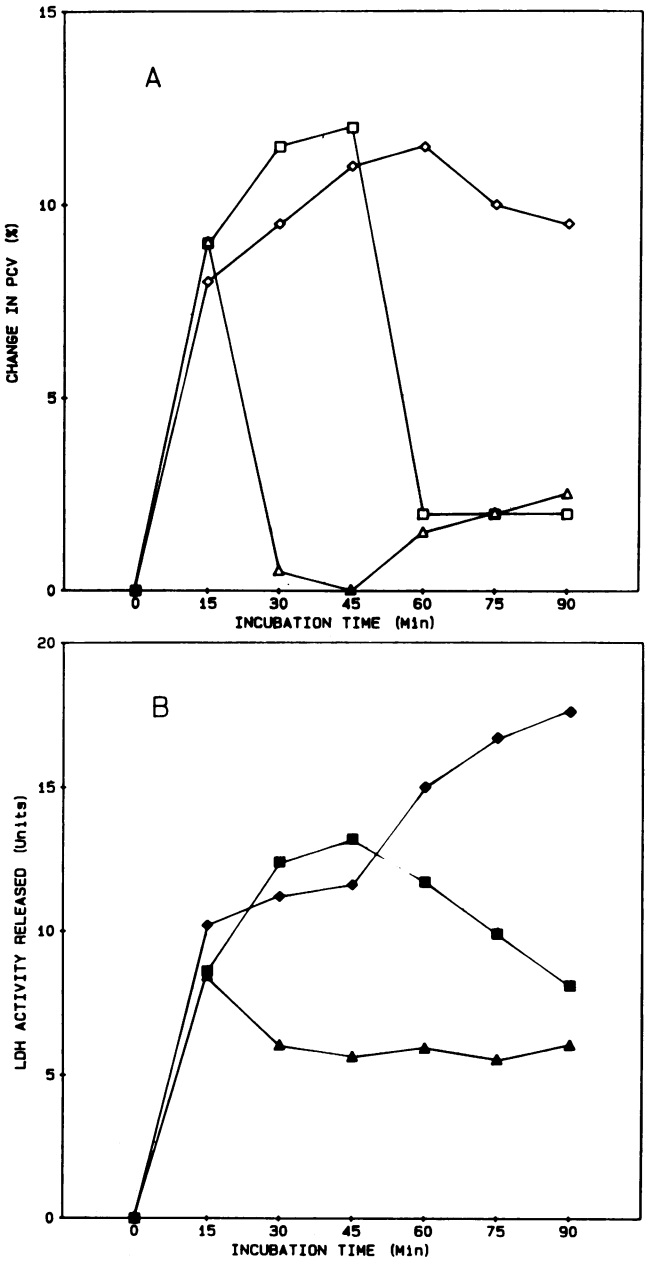

FIG. 3. Osmotic protection of BL3 cells preexposed to leukotoxin. BL3 cells (7.5×10^7) in 0.75 ml of 10 Tris–140 NaCl (pH 7.3)–1 mM $CaCl_2$ were incubated with leukotoxin (80 TU), and the PCV (A) and LDH release (B) were measured. At either 15 min (△, ▲) or 45 min (□, ■) 50% sucrose was added to yield a final concentration of 75 mM sucrose, or no sucrose was added (◇, ◆). Control cells exposed to heat-denatured leukotoxin exhibited no change during the 90-min incubation period in PCV (PCV, 6%) or LDH leakage (3.2 U). Triton X-100 released 26.6 U of LDH.

contain Ca^{2+} (Fig. 5a). Cell swelling could not be measured in these cells because they aggregated immediately upon suspension. In a parallel experiment (Fig. 5b), in which the initial incubation medium contained sucrose but no Ca^{2+}, suspension in medium lacking sucrose resulted in rapid cell swelling (PCV increased from 16 to 24%); when Ca^{2+} was included in the suspension medium, there was a slower but complete leakage of LDH (99%), but when Ca^{2+} was omitted only 40% of the cellular LDH was leaked.

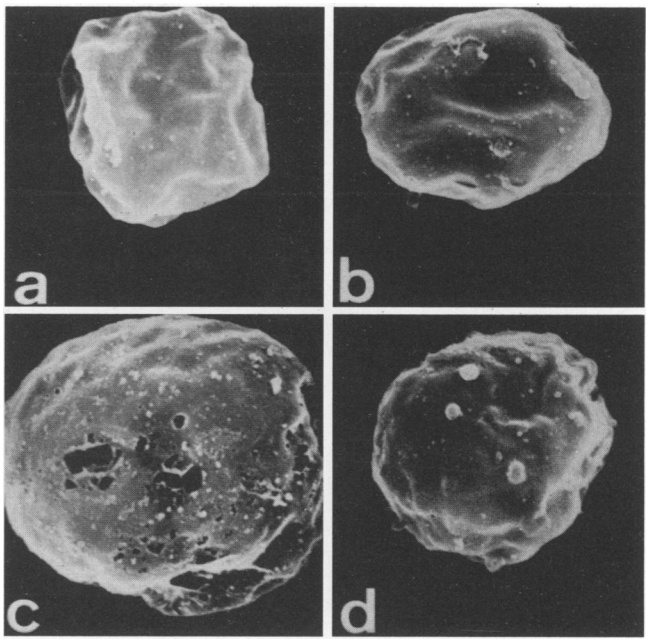

FIG. 4. Sucrose prevents cell swelling and formation of large plasma membrane defects in BL3 cells exposed to leukotoxin. Shown are scanning electron micrographs of unexposed BL3 cells in RPMI alone (a) or RPMI with 75 mM sucrose (b) and BL3 cells exposed to leukotoxin in RPMI alone (c) or RPMI with 75 mM sucrose (d) as described in Materials and Methods.

DISCUSSION

P. haemolytica leukotoxin causes rapid leakage of intracellular K^+ and subsequent colloid osmotic swelling of BL3 cells. We have shown that incubation medium made hypertonic by the addition of carbohydrate molecules with molecular weights of ≥342.3 prevented leukotoxin-induced cell swelling but not K^+ leakage from BL3 cells. We suggest, based on these findings, that leukotoxin initially forms plasma membrane pores of approximately 0.9 nm in functional diameter. This pore diameter is smaller than that estimated for pores formed by *E. coli* α-hemolysin (2 to 3 nm) or *Staphylococcus aureus* α-toxin (2 to 3 nm) with this method (5, 6). Sucrose has been shown to osmotically protect Vero cells from damage by *Clostridium perfringens* enterotoxin (13), suggesting that the functional pore size caused by this enterotoxin is similar to that of the pore formed by leukotoxin.

After leukotoxin-induced K^+ leakage and cell swelling, leukotoxin-exposed BL3 cells form large plasma membrane defects and leak LDH. Inclusion of 75 mM sucrose in the incubation medium prevented formation of large plasma membrane defects and leakage of LDH. It appears that cell swelling is required for leukotoxin-induced formation of large plasma membrane defects and LDH leakage. This conclusion is further supported by the reversal of cell swelling and cessation of LDH leakage caused by the addition of hypertonic concentrations of sucrose to cells undergoing leukotoxin-induced changes.

The effects of sucrose on cell swelling and LDH leakage had different concentration dependences. Sucrose at concentrations of ≥38 mM blocked LDH leakage, but sucrose at concentrations ≤54 mM only partially prevented cell swelling. This may indicate that a certain threshold of cell swelling is required before large plasma membrane defects

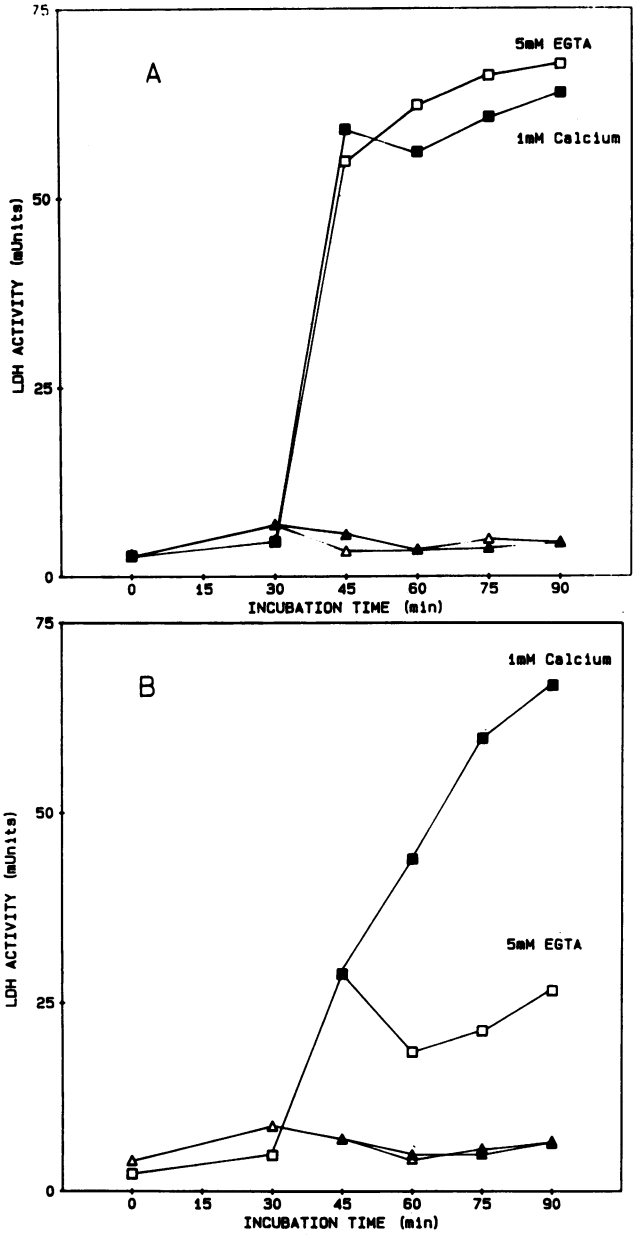

FIG. 5. Ca^{2+}-dependent steps required for leukotoxin-induced cytolysis can precede or follow cell swelling. BL3 cells (1.5×10^8) were exposed to native (■, □) or heat-denatured (▲, △) leukotoxin (900 U) in 0.75 ml of 10 mM Tris–140 mM NaCl (pH 7.3)–75 mM sucrose containing either 1 mM $CaCl_2$ (A) or 5 mM EGTA (B) for 30 min at 37°C. The cells were then collected by centrifugation (5,800 × *g* for 2 min) and washed with 10 mM Tris–140 mM NaCl–75 mM sucrose–5 mM EGTA. The washed cells were suspended in 10 mM Tris–140 mM NaCl lacking sucrose but containing either 1 mM $CaCl_2$ (■, ▲) or 5 mM EGTA (□, △). Triton X-100 (0.1%, vol/vol) released 68 U of LDH.

develop and LDH leakage occurs. The involvement of a cell swelling threshold is supported by observations of the action of *Actinobacillus actinomycetemcomitans* leukotoxin on human monocytes; the leakage of large cytoplasmic components occurs by explosive ejection of the cytoplasm rather than a gradual, continuous leakage of cytoplasmic components (17). The combination of weakened areas in the plasma membrane and increased internal hydrostatic pressure caused by cell swelling may result in sudden lysis of the plasma membrane and ejection of the cytoplasm when this threshold is exceeded.

It was previously shown that Ca^{2+} is required for certain types of leukotoxin-induced damage to BL3 cells, e.g., morphologic changes, altered vital dye uptake, decreased [^{3}H]thymidine incorporation into DNA, and leakage of LDH and ^{51}Cr (D. G. Gerbig, J. S. Foster, R. D. Walker, D. A. Bemis, and R. N. Moore. Abstr. Int. Symp. Virulent Mechanisms of Veterinary Bacterial Pathogens 1987, Iowa State University, Ames; Clinkenbeard et al., in press). All of these Ca^{2+}-dependent changes may be the direct manifestations of the formation of large plasma membrane defects that require both Ca^{2+} and cell swelling. Other bacterial cytolysins also require Ca^{2+} for their action (7, 10); however, the function of Ca^{2+} in the cytolytic activity of these toxins has not been well defined. Calcium may be required for toxin-induced phospholipase metabolism of plasma membrane phospholipids, resulting in the formation of the large plasma membrane defects, or the toxin-induced influx of Ca^{2+} may cause the disruption of the cytoskeleton, resulting in the loss of plasma membrane strength. Although both Ca^{2+}-dependent steps and cell swelling are required for *P. haemolytica* leukotoxin-induced cytolysis of BL3 cells, the temporal relationship between these steps does not appear to be important; i.e., incubation with Ca^{2+} before or concurrent with cell swelling resulted in LDH leakage.

The action of *P. haemolytica* leukotoxin on BL3 cells results in a sequence of cell-damaging events, beginning with leakage of intracellular K^+ and ending with the formation of large plasma membrane defects and loss of the cytoplasm. Cell swelling is the second event in this process, and when it is prevented, subsequent cell-damaging events are prevented from developing.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the College of Veterinary Medicine, Oklahoma State University, and by special grant number 85-CRSR-2-2618 from the U.S. Department of Agriculture.

We thank Alex Boon and Janet Durham for technical assistance.

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