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Cytotoxic Effects of Leukocidin from *Pseudomonas aeruginosa* on Polymorphonuclear Leukocytes from Cattle

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The cytotoxic action of leukocidin from *Pseudomonas aeruginosa* was studied in vitro by following the release of various intracellular markers from polymorphonuclear leukocytes from cattle (PMLC). Low-molecular markers (K^+, $^{86}Rb^+$, glucose) were lost from PMLC within 1 to 2 min after the addition of leukocytes. The release of high-molecular-weight indicators (^{51}Cr, bound to intracellular protein; lactate dehydrogenase) occurred only after swelling of the cells, leading to an increased permeability of the plasma membrane. Calcium ions stimulated the leakage of granule enzymes but retarded or inhibited the release of cytoplasmic markers. At 4 C, leukocytes were unaffected by the toxin. Leukocidin, bound at 4 C to leukocytes and treated with antiserum against leukocidin, did not damage the cells upon increasing the incubation temperature to 37 C.

Leukocidin is a cytotoxic substance produced by several strains of *Pseudomonas aeruginosa* (17). The toxin damages leukocytes from various animal species and a number of tissue cultures but is ineffective against erythrocytes and thrombocytes (18). It is a cell-bound protein with a molecular weight of about 27,500 and isoelectric points of pH 5.0 and 5.2 (18). When polymorphonuclear leukocytes from humans were exposed to leukocidin, the cells underwent a series of structural alterations and changed into enlarged, rounded ghosts with an apparently unbroken plasma membrane (19). Toxin-treated tissue culture cells (calf testicular cells, rabbit kidney cells, epithelioid green monkey kidney cells, L-cells, HeLa cells) showed a similar cytopathic effect (18). The mechanism of the cytotoxic action exerted by leukocidin on mammalian cells has not been elucidated so far.

In this paper the kinetics of the release of various intracellular markers from leukocidin-treated bovine granulocytes was studied. The results suggest that the cytotoxic effect is based on a "colloid-osmotic" process.

MATERIALS AND METHODS

Leukocidin. Leukocidin was isolated from *P. aeruginosa*, strain 158, by autolysis of washed cells (17). The toxin was purified by ammonium sulfate precipitation (20% saturation) and combined gel filtration on Sephadex G-100 superfine and Bio-Gel P-100 (18).

Buffer. If not otherwise stated, all incubations were performed in phosphate-buffered saline (PBS), pH 7.2, according to Dulbecco and Vogt (7). The buffer contained, in grams per liter: NaCl, 8.0; KCl, 0.2; KH_2PO_4, 0.2; $Na_2HPO_4 \cdot 2H_2O$, 1.25; $MgCl_2 \cdot 6H_2O$, 0.1.

Leukocytes. Polymorphonuclear leukocytes from cattle (PMLC) were prepared according to the method of Behrens and Esch (4). One volume of blood was mixed with 2 volumes of distilled water. Isotonicity was restored after 55 s by the addition of 0.5 volume of a 4.5% (vol/vol) NaCl solution, and the granulocytes were collected by centrifugation at 150 $\times g$ for 10 min. Prior to use, cells were washed twice in PBS.

^{86}Rb target cells. ^{86}Rb was chosen as an analogon of K^+ (15). PMLC (10^8) in 1 ml of PBS were incubated at 37 C for 120 min with 10 μCi of $^{86}RbCl$ (Amersham-Buchler, Braunschweig, Germany). The specific activity of the isotope used was approximately 8 to 10 μCi of Rb per μg.

After incubation, the cells were washed three times in PBS and used immediately. The normal leak rate for ^{86}Rb from target cells was about 5% of total intracellular activity when kept in PBS at 37 C for 10 min.

^{51}Cr target cells. PMLC (10^8) in 1 ml of PBS were incubated at 37 C for 30 min with 100 μCi of $Na^{51}Cr$ (delivered in 0.15 M NaCl) (Behringwerke, Marburg, Germany) and washed three times with PBS.

Measurement of leukocidic activity. To standardize leukocidic activity, the microscopic slide adhesion method (11) was used. The slides had a number of fields (0.6 mm in diameter), which were prepared by fixing a piece of punched Parafilm on the glass. Serial dilutions of toxin in PBS were incubated with human granulocytes at 37 C for 60 min in a moist chamber. One minimal leukocidic dose (MLeD) was the highest dilution of toxin that destroyed all leukocytes of a slide field (about 6,000 to 8,000 cells).

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Measurement of isotope release. Suspensions of labeled PMLC were set up in polyethylene centrifuge tubes, and all reagents except toxin were added. Unless otherwise stated, 2×10^7 PMLC/ml and 100 MLeD of leukocidin per ml were used. The tubes and the toxin were preheated to 37 C, and the test was started by adding the toxin to the tubes. Incubation was carried out in a water bath at 37 C by gentle shaking of the tubes. At various times, samples (0.2 ml) were withdrawn and centrifuged immediately at 12,000 × g for 20 s (Eppendorf-Zentrifuge, Netheler & Hinz, Hamburg, Germany). In the case of ^{86}Rb, 0.1 ml of the supernatant was transferred into a scintillation vial containing 10 ml of Unisolve 1 (Koch-Light) and counted in the ^{14}C channel of a Packard Tri-Carb liquid scintillation spectrometer. In the case of ^{51}Cr, 0.1 ml of the supernatant was measured in a Packard autogamma spectrometer. For corrections, the values of the control tubes were subtracted from the values of the appropriate experimental tubes. All tests were performed in duplicate. To permit direct comparison, all values were expressed as a percentage of total activity released from the target cells by 0.2% (vol/vol) Triton X-100.

Measurement of K^+ and Na^+. K^+ and Na^+ were determined using a flame photometer (Instrumentation Laboratories, Boston, Mass.). To determine intracellular Na^+ concentration, PMLC were sedimented by centrifugation (500 × *g*, 10 min). The supernatant was discarded, and the pellet was heated repeatedly with concentrated HNO_3 at 80 C for several hours until all cells were solubilized. Further heating resulted in evaporation of the acid, which was exchanged by distilled water. This was repeated six times, and the Na^+ contents of the solutions were determined.

Assays. β-Glucuronidase was determined after 16 h of incubation, using phenolphthalein glucuronidate as substrate (9). Cathepsin was measured according to Anson (1), with hemoglobin as substrate. Lactate dehydrogenase and glutamate dehydrogenase were estimated with reduced nicotinamide adenine dinucleotide as substrate using the Boehringer Kit (Boehringer, Mannheim, Germany), according to the suggestions of the manufacturer. Alkaline phosphatase was determined by the method of Bessey et al. (5). D-Glucose concentration was determined by the hexokinase method using the respective Boehringer Kit.

Determination of cell volume. The cell volume was either estimated by duplicate hematocrit determinations using an Adams autocrit centrifuge (Clay Adams, New York) (10) or by centrifuging the cells (500 × *g*, 10 min) in graduated centrifuge tubes.

Preparation of antiserum. Leukocidin, purified by gel filtration (18), was injected into 6-pound (ca. 2.7-kg) rabbits after a series of five subcutaneous (0.2, 0.2, 0.4, 0.6, 0.8 ml) and two intramuscular (0.6 ml each) injections at 3- to 4-day intervals. The total amount of leukocidin given was 66,000 MLeD = 1.7 mg of protein. One week after the last injection blood was collected by cardiac punction.

Reagents. Tetraethylammonium bromide was obtained from Fluka (Buchs, Switzerland). Ouabain and Triton X-100 were purchased from Serva (Heidelberg, Germany). Bovine serum albumin is a product of Behringwerke (Marburg, Germany).

RESULTS

Cytotoxic action of leukocidin as a function of time. When $^{86}Rb^+$-labeled PMLC were incubated with leukocidin at 37 C, the leakage of $^{86}Rb^+$ occurred immediately after the addition of leukocidin (Fig. 1). A maximum efflux of the intracellular $^{86}Rb^+$ (about 82%) was measured during a period of 1 to 2 min of incubation. Similar data were obtained for the toxin-mediated release of K^+ and D-glucose from PMLC (Fig. 2). When assaying the glucose concentration in the suspending medium, a second increase of extracellular glucose was detected 3 min after the addition of leukocidin to the leukocytes. This second increase may be due to the cleavage of glycogen, since the glucose concen-

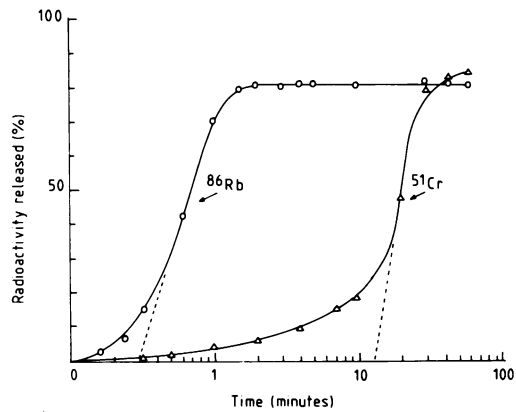

FIG. 1. *Release of $^{86}Rb^+$ and ^{51}Cr from labeled PMLC exposed to leukocidin as a function of time. Incubation at 37 C.*

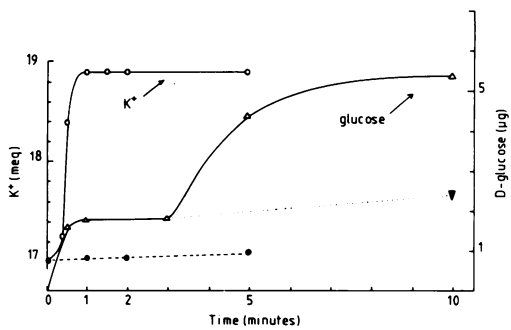

FIG. 2. *Release of K^+ and D-glucose from PMLC exposed to leukocidin as a function of time. PMLC (2×10^8 cells/ml) were incubated with leukocidin (1,000 MLeD/ml) at 37 C. Symbols: (○) K^+ (milliequivalents); (△) D-glucose (micrograms) in the cell-free supernatant of leukocidin-treated PMLC; (●) control: K^+ (milliequivalents) in the cell-free supernatant of untreated PMLC; (▼) control: D-glucose in the supernatant of sonicated PMLC.*

tration in control cells was only slightly higher than that released by leukocidin from an equivalent number of PMLC (Fig. 2).

Using ^{51}Cr-labeled PMLC as target cells, the leukocidin-mediated leakage of ^{51}Cr (Fig. 1) was much slower than that of K^+ or $^{86}Rb^+$. Extrapolation of the linear portion of the release rates indicated that ^{51}Cr was liberated after a lag period of approximately 10 min, whereas the lag period for $^{86}Rb^+$ was about 15 s. A maximum loss of intracellular ^{51}Cr (about 86%) was achieved from toxin-treated cells after 60 min of incubation at 37 C.

When studying the release of different marker enzymes from leukocidin-treated PMLC, the leakage of lactate dehydrogenase (cytoplasmic marker) and β-glucuronidase (indicator for granule enzymes) started about 10 min after the addition of leukocidin (Fig. 3). Very low glutamate dehydrogenase activity (reference for mitochondrial enzymes) was measured in the cell-free supernatant of PMLC after an incubation time of 15 min.

Cytotoxic action of leukocidin as a function of cell concentration and toxin activity. The dependence of toxin-mediated $^{86}Rb^+$ release on cell concentration and toxin activity is shown in Fig. 4a and 4b, respectively. On varying the leukocyte concentration with a constant level of leukocidin (100 MLeD/ml), the release of $^{86}Rb^+$ increased up to a cell number of 10^8 leukocytes/ml. The efflux of $^{86}Rb^+$ proceeded in a linear manner at a concentration of 2×10^7 to 8×10^7 cells/ml. The maximal release (percentage of total intracellular $^{86}Rb^+$) decreased slightly with increasing leukocyte concentration. At the incubation of 2×10^7 PMLC/ml with various concentrations of leukocidin a sigmoid relationship between leukocidin concentration and the extent of leukocidic activity was observed. With 100 MLeD of leukocidin per ml, 82% of the intracellular $^{86}Rb^+$ was released. As a result of these studies, all experiments were carried out by using 2×10^7 PMLC/ml and 100 MLeD of leukocidin per ml.

The addition of fresh leukocytes (2×10^7) to a leukocidin (100 MLeD/ml)-treated cell suspension (2×10^7 PMLC/ml) did not result in any further release of $^{86}Rb^+$.

Swelling of leukocidin-treated PMLC. It had been observed under the phase-contrast microscope that treatment of leukocytes with leukocidin causes a marked swelling of the cells (19). In Fig. 5 changes in cell volume with time and the relation of such changes to K^+ efflux are demonstrated. It shows that swelling occurred later than the leakage of K^+. Since the Na^+ ion concentration in leukocidin-treated cells was considerably higher than in normal cells (Table 1), the swelling might have been associated with the uptake of Na^+.

Cytotoxic action at 4 C. Incubation of PMLC with leukocidin for 2 h at 4 C did not cause any significant morphological alteration of the cells. However, when the cooled leukocytes were transferred into a water bath 37 C, the typical cytotoxic effect appeared immediately.

Effect of antiserum on the action of leukocidin. Leukocytes were treated with leukocidin for 60 min at 4 C and washed twice with PBS at the same temperature. Normal serum or specific antiserum against leukocidin was added to the cell suspension before increasing the incubation temperature to 37 C. The cytotoxic effect

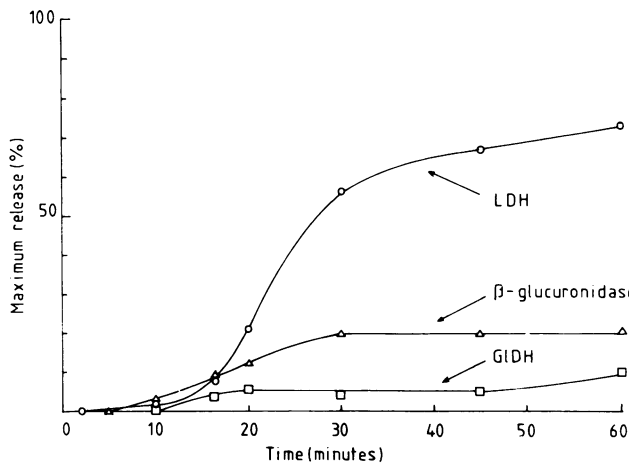

FIG. 3. *Release of lactate dehydrogenase (LDH), β-glucuronidase, and glutamate dehydrogenase (GlDH) from PMLC exposed to leukocidin as a function of time. Incubation at 37 C.*

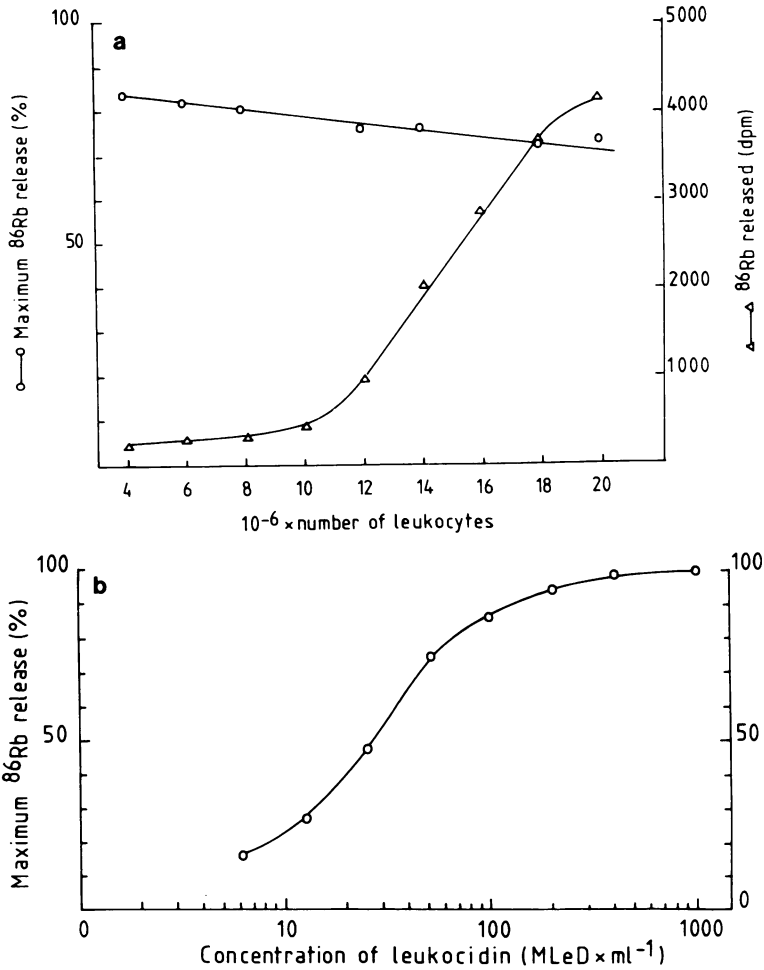

FIG. 4. *(a) Cytotoxic action of leukocidin (100 MLeD/ml) as a function of cell concentration. Incubation time, 60 min at 37 C. Symbols: (○) Release of $^{86}Rb^+$ from target cells (percentage of total intracellular $^{86}Rb^+$ activity); (△) release of $^{86}Rb^+$ from target cells (disintegrations per minute). (b) Cytotoxic action of leukocidin as a function of toxic activity. Incubation time, 60 min at 37 C. Symbols: (○) Release of $^{86}Rb^+$ from target cells (2 × 10^7 PMLC/ml) (percentage of total intracellular $^{86}Rb^+$ activity).*

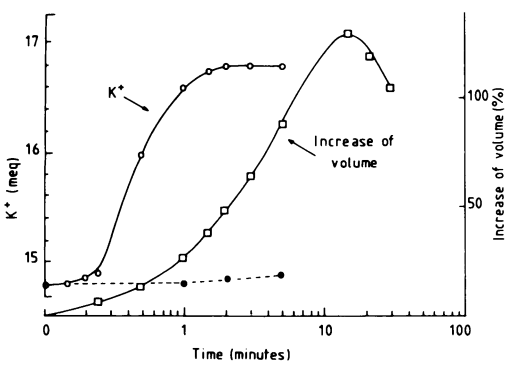

FIG. 5. *Time course of toxin-mediated swelling of cells. PMLC (2 × 10^8/ml) were incubated with leukocidin (1,000 MLeD/ml) at 37 C. Symbols: (○) K^+ (milliequivalents) in the cell-free supernatant of leukocidin-treated cells; (□) increase of volume of PMLC (percent); (●) control: K^+ (milliequivalents) in the cell-free supernatant of untreated cells.*

was completely inhibited by antileukocidin but not by the neutral serum. Two minutes after the addition of leukocidin to PMLC no antibody-mediated protection of the cells was detectable (Fig. 6). The same period was found to be necessary for a maximal efflux of $^{86}Rb^+$ from toxin-treated PMLC (Fig. 1).

Effect of calcium ions on the action of leukocidin. By observations with the phase-contrast microscope, several differences have been

TABLE 1. *Sodium uptake and swelling of PMLC treated with leukocidin*[a]

Cells tested	Na^+ (meq/ml)	Cell vol	Increase in vol (%)
Leukocidin treated	139.0	3.10	377
Control	14.2	0.65	
PBS	152.5		

[a] PMLC (1.2×10^9 cells in PBS) were mixed with 5,000 MLeD of leukocidin in a graduated centrifuge tube. After incubation (15 min at 37 C), tubes were centrifuged ($500 \times g$, 10 min) and the volume was read.

revealed in the morphology of the leukocidin-damaged leukocyte depending on the presence or absence of Ca^{2+} in the suspending medium (19). In the absence of Ca^{2+}, toxin-treated leukocytes enlarged markedly and assumed about double the size of the original cells. The granules as well as the nucleus survived for hours in the spherical swollen ghosts (Fig. 7a). In the presence of Ca^{2+} (1 mM) the leukocytes swelled to a lesser degree, their granules became clustered at the periphery of the cells, and their nuclei disappeared (Fig. 7b). In addition, with Ca^{2+} the leukocytes showed a distinct tendency for separating small vesicles from the cell (Fig. 7c).

A specific effect was exerted by Ca^{2+} on the liberation of granule enzymes from leukocidin-treated leukocytes. Cathepsin and β-glucuronidase were used as markers of the azurophilic granules (3), and alkaline phosphatase was used as a marker for the specific granules (3). In the presence of Ca^{2+} (1 mM) up to 80% of the granule-bound enzyme activities was released into the suspending medium by the action of leukocidin. When Ca^{2+} was omitted from the medium, only a maximum of 20% of the granule-bound enzyme activities was liberated (Fig. 8b and c). No quantitative difference was seen in the release of the marker enzymes from the azurophilic and specific granules. In contrast, Ca^{2+} exerted an inhibitory effect on the liberation of lactate dehydrogenase from leukocidin-treated PMLC (Fig. 8a).

Influence of different substances on the cytotoxic action of leukocidin. Tetraethylammonium bromide (0.1 M), known to block the potassium channel in nerves (2), retarded the toxin-mediated efflux of $^{86}Rb^+$ but did not inhibit it (Fig. 9). The addition of ouabain at concentrations between 10^{-5} and 10^{-3} M had no effect on the efflux of $^{86}Rb^+$ from leukocidin-treated target cells over a 1-h period.

When the leukocytes were suspended in PBS containing bovine serum albumin (100 mg/ml), the swelling of the toxin-treated cells was largely prevented (Fig. 7d) and the release of ^{51}Cr, lactate dehydrogenase, and β-glucuronidase was markedly inhibited. However, the leakage of $^{86}Rb^+$ was not influenced by this.

DISCUSSION

The rapid release of K^+ or $^{86}Rb^+$ from leukocidin-treated leukocytes could result from the following three possibilities: (i) increased permeability of the cell membrane; (ii) interference with active potassium transport; (iii) a valinomycin-like carrier effect of leukocidin. The results of the present investigation can best be accounted for by the first assumption, since the efflux of K^+ was accompanied by a simultaneous loss of glucose and was followed by the leakage of cellular proteins. In addition, the dramatic loss of K^+ would indicate that leukocidin directly increases the membrane permeability for K^+ rather than inhibiting the active transport of this ion. This presumption is supported by the results, showing that the action of leukocidin was not inhibited by ouabain and was only slightly influenced by tetraethylammonium ions. The third hypothesis (above) can be excluded, since the permeability of the membrane was increased both for K^+ and Na^+.

The cytotoxic effect brought about by leukocidin on PMLC may best be explained by the

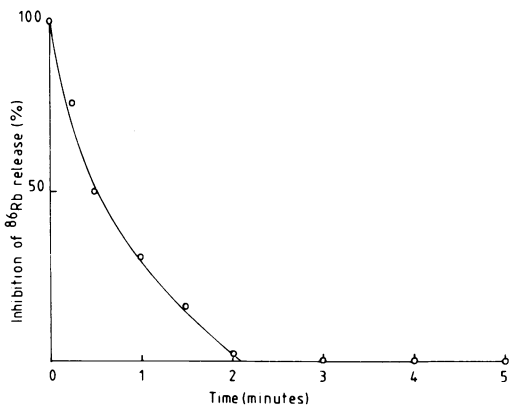

FIG. 6. *Inhibition of toxin-mediated release of $^{86}Rb^+$ from PMLC by antiserum against leukocidin. To nine centrifuge tubes, each containing 0.1 ml of $^{86}Rb^+$-labeled PMLC (4×10^7 cells/ml), 0.1 ml of leukocidin (200 MLeD/ml) was added simultaneously. Into the first tube 0.2 ml of antiserum was added at the same time as well; into the other tubes antiserum was added at the indicated times. After incubation for 20 min at 37 C, the tubes were centrifuged (12,000 × g, 20 s), and the radioactivity in the supernatant was measured.*

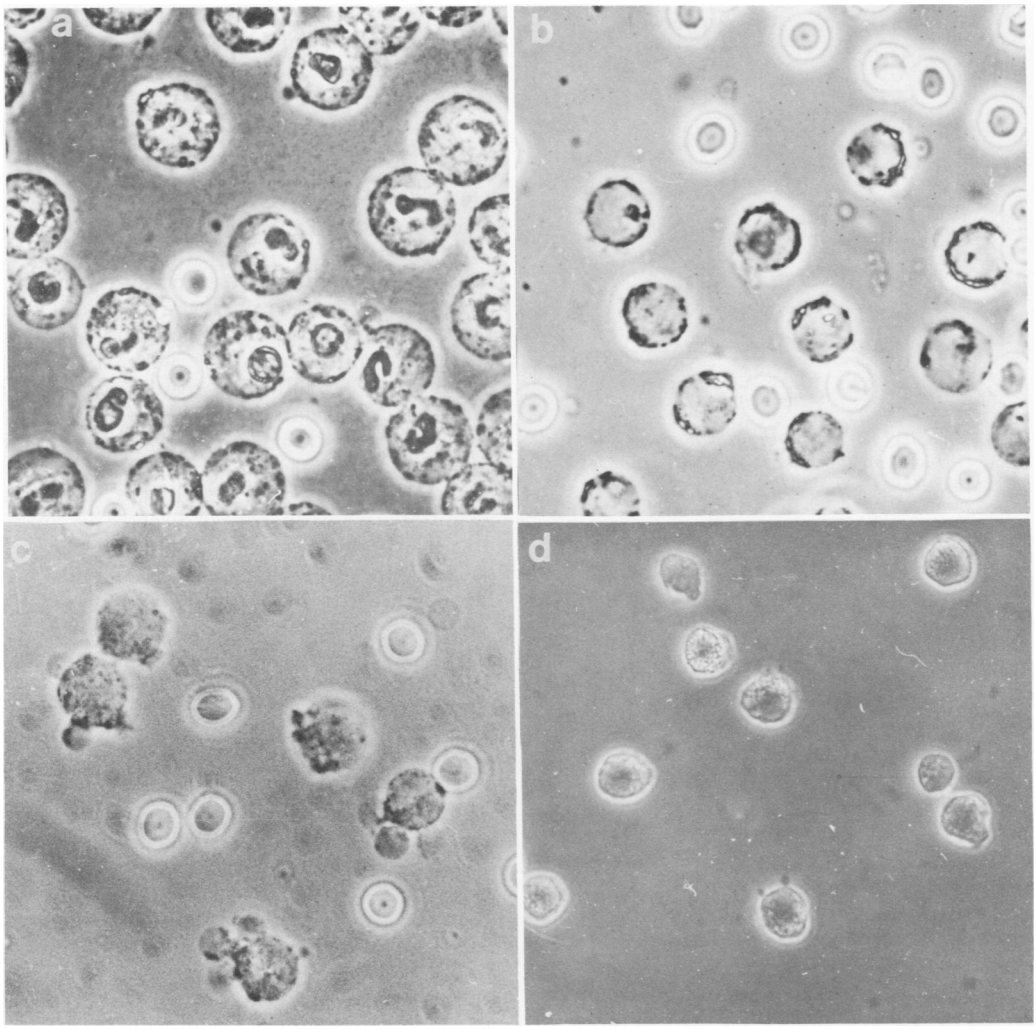

FIG. 7. *Cytotoxic effect of leukocidin to polymorphonuclear leukocytes from humans, observed by phase-contrast microscopy. Leukocytes were suspended in (a) PBS without Ca^{2+}, (b, c) PBS with 1 mM Ca^{2+}, and (d) PBS with 100 mg of bovine serum albumin per ml without Ca^{2+}.*

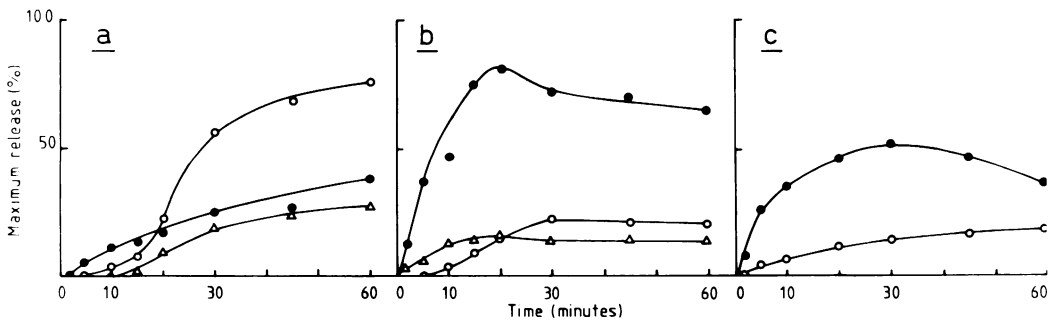

FIG. 8. *Effect of Ca^{2+} on the secretion of LDH (a), β-glucuronidase (b), and alkaline phosphatase (c) from leukocidin-treated PMLC. Suspending buffers: (○) PBS without Ca^{2+}; (●) PBS with 1 mM Ca^{2+}; (△) PBS with 100 mg of bovine serum albumin per ml without Ca^{2+}; (▲) PBS with 100 mg of bovine serum albumin per ml + 1 mM Ca^{2+}.*

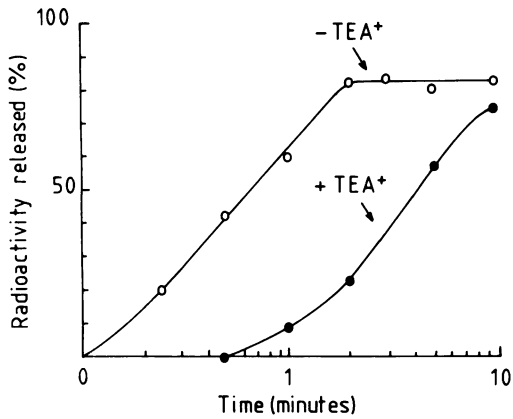

FIG. 9. *Effect of tetraethylammonium bromide (TEA) (0.1 ml) on the release of $^{86}Rb^+$ from leukocidin-treated PMLC.*

production of lesions in the cell membrane. Thus the physiological cation gradient across the membrane is disrupted and a rapid exchange of inorganic cations takes place. If the leukocytes are suspended in PBS, water enters the cells due to the colloid-osmotic pressure of the cytoplasmic macromolecules. As a result, the leukocytes swell and the lesions in the membrane expand so that larger molecules can escape from the cell: lactate dehydrogenase or ^{51}Cr, which is bound to proteins of a molecular size between 80,000 and 250,000 (14). If the leukocytes are suspended in PBS containing osmotically active macromolecules (e.g., albumin) in sufficient concentration to balance the osmotic pressure inside the cell, no swelling occurs and the leakage of cell proteins is inhibited. A similar mode of action has been described by Green et al. (12) for the complement-mediated cytotoxic effect of antitumor cell antiserum on Krebs ascites tumor cells and by Duncan (8) for streptolysin O hemolysis.

How the toxin impairs the cell membrane is unknown. Several results provide evidence that the toxic effect may not depend on an enzymatic reaction. If the rate of $^{86}Rb^+$ release was determined as a function of toxin concentration, a sigmoid curve was obtained. At low concentrations, enzymes exhibit a linear relationship between the rate of enzymatic effect and the enzyme concentration (6). Moreoever, leukocidin that had acted once on granulocytes did not damage fresh leukocytes.

On measuring the cytotoxic action of leukocidin as a function of cell concentration, the percentage of release of $^{86}Rb^+$ decreased with increasing leukocyte concentration. This result could indicate a multihit requirement for cytotoxic effect.

Tetraethylammonium ions only retarded the efflux of $^{86}Rb^+$ even when used in high concentrations. It was suggested by these observations that the loss of K^+ occurred through preformed K^+ channels to a slight degree but through lesions in the plasma membrane to a great degree.

The toxin-mediated release of granule enzymes from PMLC may be interpreted in two ways: (i) lesion of the granules, causing the release from granule substances into the cytoplasm and further through the leaky cell membrane into the surrounding medium; (ii) fusion of the granule membrane with the plasma membrane. The following results suggest that the second alternative may be correct. (i) The release of granule enzymes was stimulated by Ca^{2+}, an essential prerequisite for membrane fusion (16). (ii) As reported earlier (18), isolated granules were not damaged directly by leukocidin. (iii) If the granule enzymes had been released into the cytoplasm, they should have leaked into the suspending medium together with the cytoplasmic proteins. However, in the presence of Ca^{2+}, the liberation of granule enzymes occurred much earlier than that of the plasma proteins.

In addition, as observed by phase microscopy, toxin-treated leukocytes retained their granules when Ca^{2+} was omitted from the suspending buffer. On the addition of Ca^{2+}, the granules clustered close to the plasma membrane (Fig. 7b). Moreover, in the presence of Ca^{2+}, granulocytes revealed a special disposition to extrude and separate small vesicles from the cell (Fig. 7c). This may also indicate membrane fusion.

The extrusion of granule proteins from polymorphonuclear leukocytes by membrane fusion due to the action of staphylococcal leukocidin had been reported by Woodin and Wieneke (20). However, their results differed from our experiments in that in their studies alkaline phosphatase was secreted only at low activity.

ACKNOWLEDGMENT

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