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An *Enterobacter cloacae* toxin able to generate oxidative stress and to provoke dose-dependent lysis of leukocytes

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Abstract

We investigated an *Enterobacter cloacae* strain exhibiting high hemolytic and leukotoxic activity. Monomeric and polymeric forms of the toxin showed similar effects on blood cells, although the polymer was more active than the monomer. Fluorescence microscopy revealed that both forms of the FITC-labeled toxin interacted with leukocytes, principally with neutrophils. Prelytic concentrations of polymeric and monomeric toxin significantly increased the production of reactive oxygen species (ROS) in neutrophils. Conversely, lytic concentrations of both toxin forms showed an increase followed by a decrease of ROS due to neutrophil damage. Monocytes did not show oxidative stress at all the toxin concentrations assayed. The toxin–neutrophil interaction at prelytic concentrations of toxin-stimulated ROS production and led to oxidative stress with subsequent cell death by apoptosis. However, high concentrations of *E. cloacae* toxin damaged leukocytes, producing lysis before the trigger of apoptosis, which suggests that the toxic effect is concentration dependent. The inhibition of oxidative stress observed with genistein and chloroquine suggests a potential involvement of the tyrosine kinase and nitric oxide synthesis pathways in *E. cloacae* toxin-mediated elevation of ROS.

Keywords: *Enterobacter cloacae*; RTX toxins (repeats in the structural toxin); Leukocytes; Oxidative stress; Endocytosis; Apoptosis

Introduction

The superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^\cdot$), hypochlorous acid (HOCl), and nitric oxide ($NO^\cdot$) are formed by physiological processes in macrophages. These reactive oxygen species (ROS) and reactive nitrogen intermediates can also arise under certain pathological conditions. During infectious diseases, ROS are produced within phagosomes at high enough concentrations to kill bacteria (Miller and Britigan, 1997). Conversely, high levels of ROS can alter the physiology of neutrophils and eventually exert lytic action on these cells. Furthermore, some bacterial toxins have the capacity to undergo redox cycling with ROS increase and subsequent leukocyte lysis (King et al., 1999; Colin and Monteil, 2003).

ROS can be studied by luminol amplified chemiluminescence (CL), which allows to investigate the effect of bacterial toxins on cells, including leukocytes and other phagocytes (Bhakdi and Martin, 1991).

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Exposure of leukocytes to toxins can lead to lysis by membrane damage or to functional alterations such as the change of oxidative metabolism that can finally produce death. The latter may be an apoptotic process, which was described for viral and some bacterial pathogens (Muller and Rudel, 2001) and their leukotoxins (Narayanan et al., 2002).

Certain RTX toxins (repeats in the structural toxin) are pore-forming proteins and induce apoptosis in susceptible cells (Lally et al., 1999). Binding to the target cell surface represents the first step in the initiation of this process. The downstream events leading to toxin-induced apoptotic cell death are being identified (Frey and Kuhnert, 2002). Leukotoxin-induced apoptosis could be the principal mechanism by which ROS are liberated during oxidative stress of leukocytes. Moreover, molecular and biochemical mechanisms of apoptosis can be accompanied by necrotic cell death (Korostoff et al., 2000).

Enterobacter cloacae is an opportunistic pathogen associated with different affections, including patients with burns, urinary infections, pneumonia, bacteremia, and nosocomial infection outbreaks of septicemia in neonatal intensive care (Oana et al., 2000; Yu et al., 2000). Toxins of *E. cloacae* are still not clearly defined at present; it seems that some metabolites of these bacteria may play a prominent role in infections. Although little is known about toxins of *E. cloacae*, probably one of the most important determinants of virulence is a cytotoxin similar to Shiga-like toxin II (Paton and Paton, 1996).

According to previous research performed in our laboratory, a leukotoxic and hemolytic toxin was purified from clinical isolates of *E. cloacae* by $(NH_4)_2SO_4$ precipitation, dialysis, chromatography by gel filtration, and HPLC. Data on the purity and nature of this toxin indicated that lipopolysaccharide was extracted from the protein during purification (Barnes et al., 1997). SDS-PAGE electrophoresis showed more than one band indicating that the monomeric toxin is able to form polymers and aggregates like some RTX toxins (Welch, 2001). Further, *E. cloacae* toxin was identified by genetic determination and it was possible to find hybridization with DNA of *Escherichia coli* hemolysin (HlyA); and the toxin gene presented a DNA segment encoding a glycine-rich amino acid domain similar to HlyA (Barnes et al., 2001a). Some aspects of the leukotoxic mechanism of action were described: the monomeric toxin induced oxidative stress in neutrophils as revealed by ROS measurements (Albesa et al., 2000; Barnes et al., 2001b).

The present investigation was performed to determine the dose-dependent capability of the *E. cloacae* toxin to generate oxidative stress and to provoke lysis and induction of apoptosis in leukocytes, using a new strain with important hemolytic and leukotoxic activity and a significant tendency to form polymers.

Materials and methods

Production and purification of *Enterobacter cloacae* toxin

Among strains isolated from patients with infections, the *E. cloacae* strain E_2 was selected because of its hemolytic capacity. The identification was done with a computerized program for clinical strains of Gram-negative, oxidase-negative, aerobic bacteria (Sensident EM-Ident E). The toxin was purified from a culture of E_2 in trypticase soy broth (TSB) grown for 18 h at 37 °C. Then, 0.18 M 2-mercaptoethanol (2-ME) was added and incubated for 30 min at 37 °C. The culture supernatant (500 ml) was obtained by centrifugation at 13,000*g* for 30 min at 4 °C. The toxin was precipitated with 50% (p/v) $(NH_4)_2SO_4$ at 4 °C overnight, centrifuged for 30 min at 16,000*g* and dialyzed for 48 h at 4 °C against 0.01 M phosphate buffer (pH 6.8). Chromatography in Sephadex G 100 (40 × 2 cm column) was performed with phosphate-buffered saline (PBS; pH 7.2). Subsequently, reverse-phase high-performance liquid chromatography (HPLC) in a Konik 500 G instrument with a TSK G 3000 PW column (7.5 mm inner diameter × 300 cm, Varian MicroPak) and UV detector (UVIS-200) was performed. Elution was performed at 0.7 ml/min with a linear gradient of solution A (30% acetonitrile and 0.12% trifluoroacetic acid) and solution B (65% acetonitrile and 0.1% trifluoroacetic acid). The absorption was measured at 280 nm. Monomers were separated from polymers by gel filtration in Sephadex G 100 and HPLC. Toxin preparations were subjected to electrophoresis in 13% sodium dodecyl sulfate (SDS)-polyacrylamide gels, which were stained with Coomassie blue. Different molecular markers were applied for each electrophoresis: lactalbumin (14.2 kDa), carbonic anhydrase (28.5 kDa), glyceraldehyde-3-phosphate dehydrogenase (35.9 kDa), ovalbumin (45.0 kDa), phosphorylase b (96.4 kDa), β-galactosidase (113.6), and myosin (198.1 kDa).

Toxin binding assay

FITC (1 mg/ml) in 1 M sodium carbonate at pH 9.6 was incubated with 10 mg/ml monomeric or polymeric *E. cloacae* toxin (FITC stock/toxin 10:1; v/v) for 1 h. Then, the mixture was dialyzed for 18 h against PBS. Leukocytes and erythrocytes were washed in PBS and incubated with FITC-labeled toxin (0.04 and 50 μg/ml) for 30 min on glass slides. After being washed three times, the slides were stained with Evans blue. Fluorescence microscopy was employed to detect toxin binding.

Leukotoxic activity

The human leukocytes (HL) were obtained from heparinized blood of healthy individuals according to the protocol of our University Human Ethics Committee. HL were concentrated in 6% dextran for 60 min at 25 °C. After separation, hypotonic lysis of the remaining erythrocytes was applied and followed by centrifugation in a Ficoll-Hypaque gradient. Ficoll-Hypaque was employed to separate neutrophils from monocytes. The HL were collected, washed twice and suspended in Hanks' balanced salt solution (HBSS) pH 7.2 with 5% bovine serum albumin and without phenol red (10^6 cells/ml). Cells were stored on melting ice and used within 4 h. Cell counts were performed with Turks staining. Cytotoxicity was assessed by Trypan blue exclusion (Kuhnert et al., 2003). HL suspensions were incubated at different concentrations of the HPLC-purified toxin for 30, 60, 120, and 480 s at 37 °C. The relation HL/μg toxin/ml varied from 10^6/0.004 to 10^6/50 μg/ml.

Chemiluminescence assay

The oxidative metabolism of neutrophils and monocytes was assayed by CL, based on the oxidation of luminol by ROS (Becerra and Albesa, 2002). The CL assay was performed in polypropylene tubes containing 0.1 ml of neutrophils or monocytes (10^6/ml), 0.1 ml luminol (3.36 μM) and 0.1 ml of different concentrations of purified toxin (0.004–50 μg/ml) without 2-ME in a final volume of 0.5 ml of HBSS. The spontaneous CL was determined by incubating neutrophils or monocyte in HBSS without toxin. The CL background of each vial was checked before using it. CL was measured at room temperature in a BioOrbit model 1253 luminometer. The light emission at different times was expressed as relative unity of light (RUL) per 10^6 cells, with subtraction of the background. Phorbol myristate acetate (PMA) (0.1 mg/ml) was used as positive control and heat-inactivated (100 °C) toxin as negative control (Barnes et al., 1997).

Genistein (0.1 mM), an inhibitor of protein tyrosine-phosphorylation, was prepared in PBS (pH 6.8) and incubated with 10^6 leukocytes in presence of 0.004–0.4 μg/ml of monomeric or polymeric *E. cloacae* toxin plus luminol (3.36 μM). The final volume was adjusted to 0.5 ml HBSS. A similar assay was performed with 0.05 mM chloroquine, a phagosome pH-increasing substance and an inhibitor of NO synthesis in macrophages (Hrabak et al., 1998).

Electron microscopy

Ultrastructural alterations of leukocytes were investigated after 4 h of incubation with monomeric or polymeric toxin. The ratio of HL/μg toxin/ml varied from 10^7/0.4 μg/ml to 10^7/50 μg/ml. The cells were fixed by immersion in 1% glutaraldehyde diluted in 0.1 M cacodylate buffer (pH 7.3); then, they were post-fixed in 1% OsO_4, dehydrated and embedded in araldite. Thin sections were cut in a Porter-Blum MT2 ultramicrotome and examined in a Zeiss 109 electron microscope (Zeiss, Oberkochen, Germany). Photographs were obtained using a Kodak electron imaging film.

Statistical methods

The results of biological assays were expressed as the mean values ± S.E.M. Data were analyzed using Student's t test and $p < 0.05$ was considered significant.

Sources for reagents

Trypticase soy broth (Britania); 2-mercaptoethanol, Sephadex G-100, FITC, dextran, bovine serum albumin, Ficoll-Hypaque, luminol, genistein, chloroquine (Sigma); acetonitrile, trifluoroacetic acid, sodium carbonate, RPMI 1640, Tris-HCl, sodium dodecyl sulfate, EDTA (Merck); Trypan blue (Mallinckrodt), ethanol (Porta). Chromogenic test (BioWhitaker). NaCl (Cicarelli).

Results

HPLC analysis of the toxin fraction purified from *E. cloacae* strain E_2 revealed a higher amount of polymer compared with the E1 strain; the monomer eluted at Ve 98–120 ml and the polymer at Ve 40 ml (Fig. 1). The purity of the toxin fractions was investigated by electrophoresis. The monomeric 13.3 kDa form, and the polymeric 27, 42 and 66 kDa

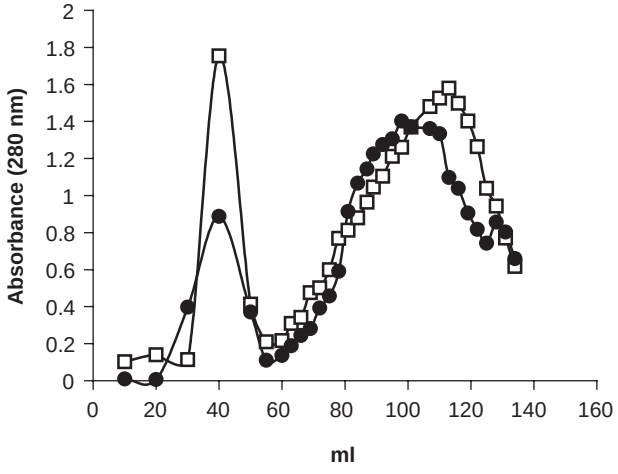

Fig. 1. Sephadex G-100 chromatographic profile of *Enterobacter cloacae* leukotoxins purified from strain E_1 (●) and E_2 (□).

forms as well as a high-mass toxin (Fig. 2, lane b) were detected.

The leukotoxicity assays of monomeric and polymeric toxin revealed that neutrophils lysed at a ratio of cells/toxin ranging from 10^6/0.4 μg/ml to 10^6/50 μg/ml. Lower toxin concentrations (10^6 cells/0.004 μg/ml to 0.04 μg/ml) were required to study the prelytic effect on oxidative metabolism of neutrophils.

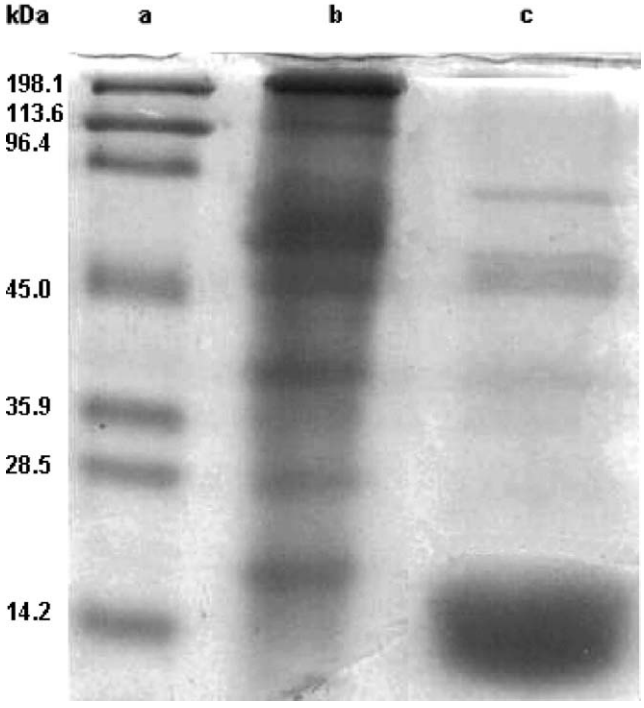

Fig. 2. SDS-polyacrylamide (13%) gel electrophoresis (Coomassie blue staining). Lane a: molecular weight marker. Lane b: Toxin sample showing different oligomeric forms (27, 42 and 66 kDa as well as a high-mass toxin). Lane c: monomeric toxin sample (13.3 kDa).

CL assays showed that 0.04 μg/ml of monomer augmented the production of ROS in a time-dependent manner (Table 1). A tenfold increase in toxin concentration (0.4 μg/ml) led to higher levels of ROS after 30 and 60 s than 0.04 μg/ml, but after 120 s ROS decayed. Conversely, concentrations of 20 and 50 μg/ml of monomer generated less ROS (Table 1), due to cell damage and lysis. The polymeric toxin was more efficient in stimulating oxidative stress than the monomer, but without a progressive augmentation of ROS due to being more harmful to leukocytes compared with the monomer (Fig. 3). Monocytes did not show oxidative stress at all concentrations studied.

Exposure of HL to FITC-labeled toxin (0.4 μg/ml) revealed toxin binding to neutrophils (Fig. 4, upper panel), but HL exhibited lysis when they were exposed to 50 μg/ml FITC-labeled toxin (Fig. 4, lower panel). The intensity of fluorescence was higher in neutrophils than in monocytes, and erythrocytes were the least labeled.

The effects of monomeric and polymeric toxin on ROS levels were counteracted by genistein or chloroquine (Table 2). Both inhibitors per se did not cause oxidative stress.

HL exposed to low concentrations of *E. cloacae* leukotoxin exhibited many of the morphologic hallmarks of apoptosis including chromatin condensation, nuclear membrane blebbing and cytoplasmic vacuolation, with intact plasma membranes (Fig. 5, upper panel). HL incubated with *E. cloacae* monomeric toxin at a high concentration presented ultrastructural

Table 1. Alterations of reactive oxygen species (ROS) production in neutrophils incubated for different times with different concentrations of monomeric and polymeric *Enterobacter cloacae* leukotoxin

Toxin fraction	Concentration (μg/ml)	30 s	60 s	120 s	480 s
Heat inactivated		0.33±0.05	0.34±0.02	0.33±0.01	0.38±0.01
Monomer					
	0.004	1.11±0.07	1.23±0.08	2.10±0.05	2.01±0.09
	0.04	3.81±0.04	6.03±0.07	9.90±0.01	9.99±0.05
	0.4	4.91±0.05	7.23±0.05	9.10±0.06	3.80±0.08
	4	5.12±0.09	4.89±0.08	4.49±0.04	4.99±0.04
	20	2.24±0.01	2.33±0.04	2.23±0.09	2.57±0.09
	50	0.90±0.03	0.93±0.05	0.91±0.01	0.98±0.02
Polymer					
	0.004	1.32±0.06	1.44±0.05	1.93±0.02	1.90±0.06
	0.04	5.22±0.07	5.34±0.09	5.93±0.04	5.03±0.08
	0.4	4.52±0.02	4.23±0.08	4.03±0.05	4.00±0.05
	4	2.19±0.04	2.18±0.10	2.13±0.09	2.03±0.05
	20	2.13±0.05	2.10±0.01	2.02±0.04	1.92±0.04
	50	0.53±0.05	0.49±0.03	0.50±0.01	0.51±0.08

ROS levels are given as RUL per 10^6 cells (see Materials and methods; Chemiluminescence assay).

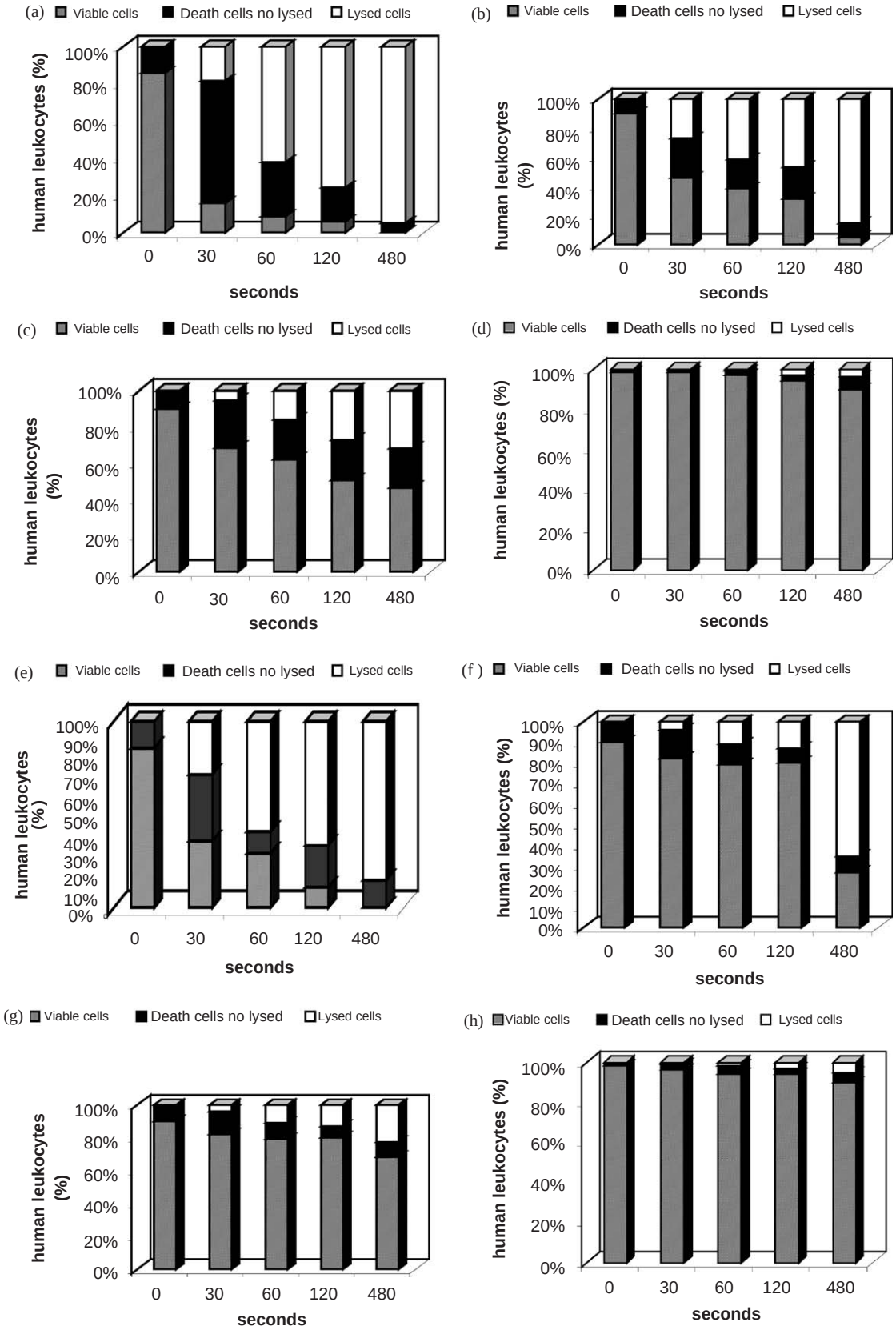

Fig. 3. Cytotoxicity as assessed by Trypan blue exclusion in HL treated with four concentrations of polymeric (a–d) and monomeric (e–h) *E. cloacae* toxin. (a, e) 50 μg/ml; (b, f) 0.4 μg/ml; (c, g) 0.04 μg/ml; (d, h) 0.004 μg/ml.

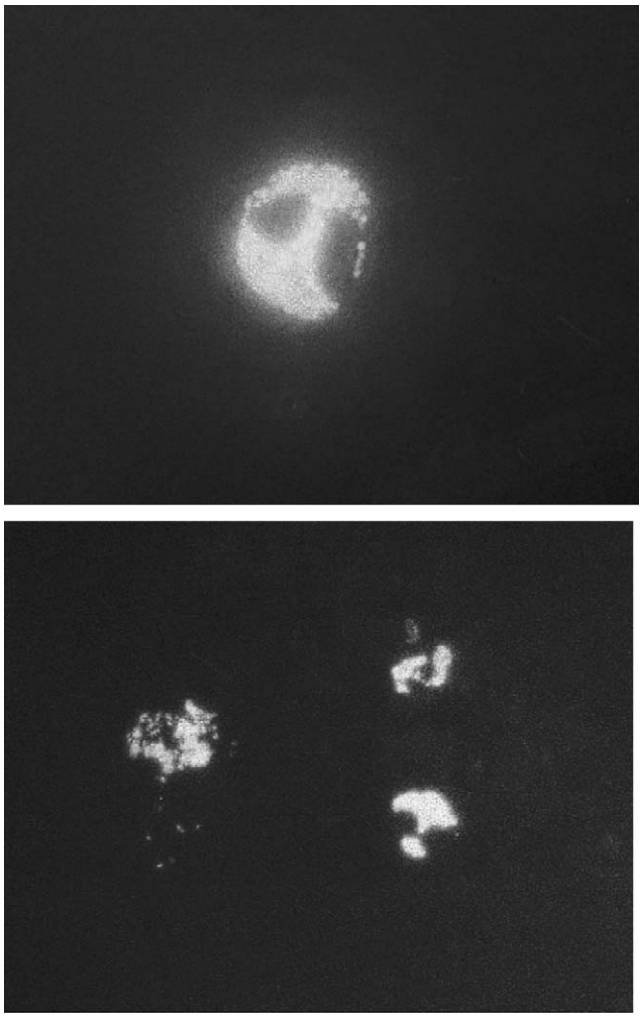

Fig. 4. Fluorescence images of human leukocytes treated with FITC-labeled toxin (0.4 μg/ml), showing intense fluorescence (upper panel), and with 50 μg/ml FITC-labeled toxin, exhibiting lysis (lower panel).

evidence of cytoplasmic and nuclear membrane rupture and swelling (Fig. 5, middle panel). Incubation of HL with the toxin at extremely low concentrations did not alter their ultrastructure. Toxin treatment of HL induced DNA fragmentation with typical internucleosomal DNA laddering (data not shown). Electron microscopic images of human leukocytes exposed to low concentrations of monomeric toxin revealed about 75% of apoptotic cells.

Discussion

The new strain of *E. cloacae*, E_2, investigated here exhibited higher hemolytic and leukotoxic activity than the strain E_1 initially studied (Barnes et al., 1997). Moreover, the latter showed less polymeric toxin than E_2. Strains E2 and E1 showed identical physiochemical properties, i.e. a molecular weight of 13 kDa for the monomer and susceptibility to calcium (Barnes et al., 2001a).

The stimulation of ROS production by certain bacterial toxins can lead to cell destruction (Wang et al., 1998). This seemed to be the case for the monomeric and polymeric forms of *E. cloacae* toxin. Both forms of toxin stimulated ROS at prelytic concentrations, but high concentrations caused neutrophil lysis. This dose-dependent leukocyte response to *E. cloacae* toxin resembled the characteristics observed in lymphocytes incubated with *Pasteurella haemolytica* leukotoxin (LKT). Low concentrations of LKT induced apoptosis and high concentrations induced oncotic cell lysis in bovine lymphocytes (Sun et al., 2000).

Electron and fluorescence microscopy showed that monomeric (Figs. 4 and 5) and polymeric (data not

Table 2. Inhibition of *E. cloacae* toxin-induced oxidative stress in neutrophils by genistein (0.1 mM) and chloroquine (0.05 mM)

Treatment	*E. cloacae* toxin concentration (μg/ml)		
	0.4	0.04	0.004
Monomer	9.10 ± 0.06	9.90 ± 0.01	2.10 ± 0.01
Monomer + genistein	1.83 ± 0.01	1.98 ± 0.05	1.79 ± 0.02
HBSS + genistein	0.23 ± 0.01	0.25 ± 0.06	0.21 ± 0.03
Monomer + chloroquine	1.03 ± 0.08	1.11 ± 0.05	1.15 ± 0.07
HBSS + chloroquine	0.24 ± 0.04	0.28 ± 0.03	0.26 ± 0.07
Heat-inactivated toxin	0.33 ± 0.05	0.39 ± 0.01	0.30 ± 0.02
Polymer	4.03 ± 0.05	5.93 ± 0.04	1.93 ± 0.04
Polymer + genistein	0.99 ± 0.02	1.19 ± 0.05	1.29 ± 0.01
HBSS + genistein	0.28 ± 0.04	0.23 ± 0.01	0.20 ± 0.06
Polymer + chloroquine	0.87 ± 0.03	1.27 ± 0.07	1.07 ± 0.06
HBSS + chloroquine	0.19 ± 0.05	0.15 ± 0.06	0.29 ± 0.07
Heat-inactivated toxin	0.31 ± 0.03	0.32 ± 0.03	0.28 ± 0.05

ROS levels are given as RUL per 10^6 cells after treatment with toxin for 120 s (see Materials and methods; Chemiluminescence assay).

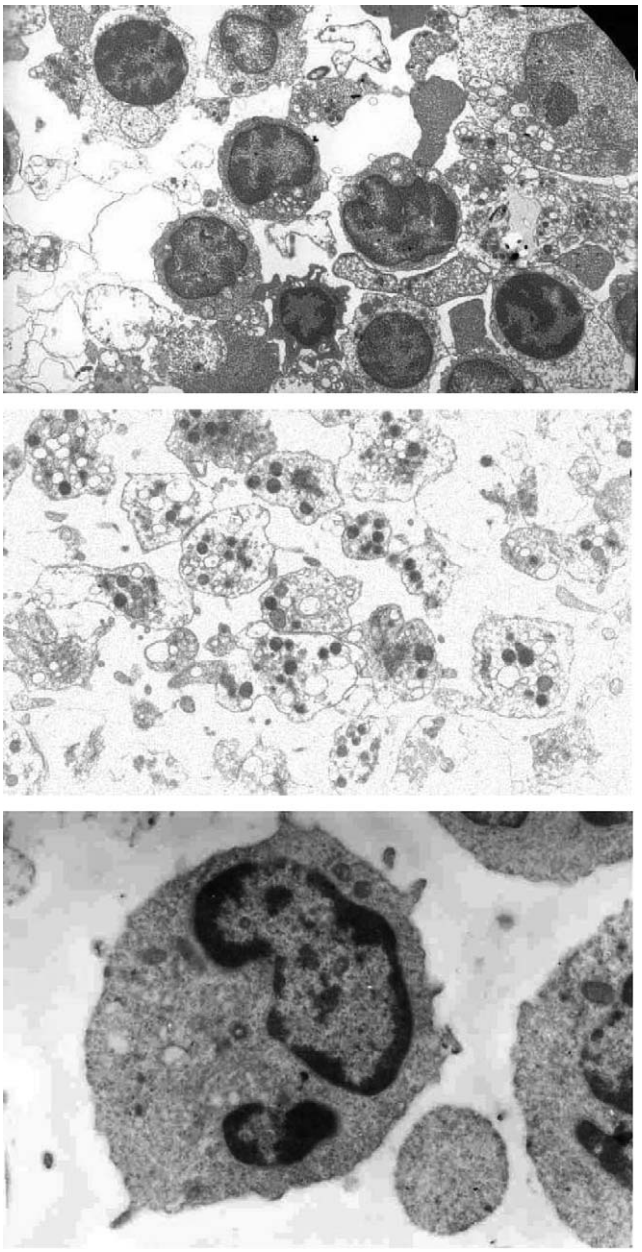

Fig. 5. Electron microscopic images of human leukocytes exposed to *E. cloacae* monomeric toxin. Upper panel: after treatment with a low concentration of toxin (0.4 μg/ml) HL exhibited many of the morphologic hallmarks of apoptosis including peripheral condensation of nuclear DNA, plasma membrane blebbing and cytoplasmic vacuolation with intact plasma membranes (6000 ×). Middle panel: HL incubated with *E. cloacae* monomeric toxin at a high concentration (50 μg/ml) presented ultrastructural evidence of cytoplasmic and nuclear membrane rupture and swelling (5000 ×). Lower panel: untreated control (24,000 ×).

shown) toxin interacted with leukocytes, principally with neutrophils. Monomeric and polymeric forms of toxin showed similar effects on ROS levels in blood cells, although the polymer was more effective than the monomer.

In the present study, 4 ng of polymeric toxin (0.1 ml of 0.04 μg/ml) did not provoke much lysis, but led to only prelytic effects on leukocytes. *E. coli* RTX toxin seems to be more active as 0.1 ng toxin caused lysis of erythrocytes, a cell less sensitive than leukocytes (Pellett and Welch, 1996).

The reduction of oxidative stress observed with genistein and chloroquine suggests a potential involvement of tyrosine kinase and NO synthesis pathways, respectively, in *E. cloacae* toxin-mediated elevation of neutrophil ROS levels.

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