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Pore formation, polymerization, hemolytic and leukotoxic effects of a new *Enterobacter cloacae* toxin neutralized by antiserum

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Summary

A new toxin of *Enterobacter cloacae* was purified and studied by SDS-PAGE electrophoresis with the purpose of investigating its ability to generate polymers and their molecular mass. Monomer of 13.3 kDa and structures of multimeric mass were detected. The toxin of 66 kDa was the most abundant form of toxin. This polymer and the monomer were selected to examine blood cells damage. Membrane pores caused by both toxin forms seemed to be of similar dimension (estimated in 3.6 nm by experiments with osmotic protectors) and were able to lyse erythrocytes and leukocytes. The results obtained indicate that polymerization and pore formation are involved in the molecular events that participate in the cytotoxic effects of *E. cloacae* toxin. Immunization of rabbits with 13.3 kDa toxin generated antibody response capable of inhibiting oxidative stress as well as hemolytic and leukotoxic effects. Immunoblotting indicated that monomer and polymer reacted with antihemolysin serum. The importance of *E. cloacae* toxin ''in vivo'' was studied in animals by means of assays performed in peritoneum of rats, inoculated with the hemolytic strain (C_1) and a non-hemolytic variant (C_4). Both strains stimulated infiltration of leukocytes in peritoneum, but C_1 caused cell death and lysis wheras assays with C_4 maintained the viability of leukocytes even within 5 h after extraction of samples.

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Introduction

Enterobacter cloacae is an opportunistic pathogen that causes different infections (Oana et al., 2000; Yu et al., 2000). Toxins of *E. cloacae* still have not been clearly defined so far; it seems that some metabolites of these bacteria may play a prominent role in infections. According to previous research performed in our laboratory, strain E_1 and E_2 of this bacterium can produce a hemolysin with leukotoxin activity. This toxin was purified from clinical isolates of *E. cloacae* by means of precipitation, dialysis, gel filtration and high-pressure liquid chromatography and it maintained its hemolytic and leukotoxic activity. Data on the purity and nature of this toxin indicated that it is a glycoprotein capable of lysating human, rabbit, mouse and horse erythrocytes. Trypsin treatment and heat reduced the lytic and toxic activities. The pH effect on the cytotoxin was evident and it was produced during logarithmic phase. Lipopolysaccharide was extracted from the protein during purification (Barnes et al., 1997). In addition, cross-reaction with international standard of *Escherichia coli* hemolysin (HlyA) was found by immunoblotting; however, *E. cloacae* toxin did not cross-react with hemolysin HmpA of *Proteus*. Moreover, *E. cloacae* lysin presented a glycine-rich DNA sequence similar to HlyA, and a high amount of acid and lipophilic amino acids (Barnes et al., 2001a). Some aspects of leukotoxic mechanism of action were described. *E. cloacae* toxin induced oxidative stress in neutrophil at low leukotoxin concentration, changing the kinetic of generation of reactive oxygen species (ROS) (Paraje et al., 2002). Conversely, when the increase in ROS was high, they could alter the physiology of neutrophils and eventually exert lytic action on these cells. Stimulation of oxidative stress was observed and the production of reactive ROS was measured by nitroblue tetrazolium and chemiluminescence assays (CL) (Albesa et al., 2000; Barnes et al., 2001b).

ROS could be studied by luminol amplified CL, for that reason, cellular and molecular investigations about oxidative stress could be carried out by this method. CL was applied to know the effect of bacterial toxins on cells, including leukocytes and other phagocytes (Bhakdi and Martin, 1991). The autoregulation of respiratory burst, as evidenced in peritoneal macrophages of mouse (Fernández et al., 1999), is affected by various factors. Some bacterial toxins have the capacity to undergo redox cycling with ROS increase and subsequent lyse of leukocytes (King et al., 1999; Colin and Monteil, 2003).

The level of toxins production by bacteria may determine the ability to survive host defenses or cause infections. In general, the concentration of active leukotoxins is an important virulence factor (Burriel and Dagnall, 1991; Narayanan et al., 2002). The toxic effect of certain bacterial lysins was related to alterations of membrane with different-size pores, which are formed by polymeric binding of toxin monomers on cell surfaces (Sathyamoorthy et al., 1997). In addition, oligomerization on mammalian cells seems to be involved during infection of toxic bacteria as part of mechanism of action observed in hemolysins and leukotoxins (Singh et al., 1998). This characteristic associated with the property of self-aggregation was studied in the hemolysin HlyA of *E. coli* by means of urea treatment (Soloaga et al., 1998).

In an attempt to counteract the effect of bacterial toxins, the capacity of certain hemolysins to generate neutralizing antibodies, the cytotoxic action was studied, and some of these antibodies were observed to ensure protective action against the damage caused by the infection. Antiserum against toxins has immunoprophylactic potential because bacterial toxin can be neutralized by specific antibodies before inducing cell alterations in the host. Immune sera play a protective role against infections in which toxins contribute to the development of tissue lesions, such as enterohemorhagic effect of *E. coli*-mediated colitis and hemolytic-uremic syndrome in humans (Mukherjee et al., 2002).

The purpose of our study was to explore the effect of some metabolic mechanisms of action of *E. cloacae* toxin produced by a strain with high hemolytic and leukotoxic activity. The capacity of polymerization, the size of pores generated and the toxicity ''in vivo'' were investigated in order to know whether antiserum against the toxin could neutralize the different aspects of toxin action.

Materials and methods

Purification and characterization of the toxin: An *E. cloacae* strain (E_2) was selected because of its hemolytic capacity among strains isolated from patients with infections. This *E. cloacae* strain was typified with a computerized program for clinical strains of Gram-negative oxidase-negative aerobic bacteria (Sensident EM-Ident E). The toxin was isolated from a culture of E_2 strain in 500 mL trypticase soy broth (TSB) for 18 h at 37 °C. After growth, 0.18 M 2-mercaptoethanol (2-ME) was added and incubated for 30 min at 37 °C. Cells

were separated by centrifugation at 13,000*g* for 30 min at 4 °C. The toxin was precipitated with 50% (w/v) $(NH_4)_2SO_4$ at 4 °C overnight, centrifuged at 16,000*g* for 30 min and dialysed for 48 h at 4 °C in 0.01 M phosphate buffer (PBS) pH 6.8. Proteins were chromatographied in Sephadex G 100 (40 × 2 cm) in the same buffer. Subsequently, reverse-phase high-performance-liquid chromatography (HPLC) in a Konik 500 G instrument with a TSK G 3000 PW column (7.5 mm i.d. × 300 cm, Varian MicroPak) and UV detector (UVIS-200) were realized. Elution was performed at 0.7 mL/min with linear gradient elution of Solution A (30% acetonitrile and 0.12% trifluoracetic) and solution B (65% acetonitrile and 0.1% trifluoracetic). The absorption was measured at 280 nm. Hemolytic and leukotoxic activities of chromatographic peaks were assayed and protein was measured by Bradford reaction.

Investigation of monomer and olygomers: Three pore sizes of polyacrilamide electrophoresis (PAGE: 8%, 10% and 13%) were assayed to reveal all oligomeric forms of toxin. Sodium dodecyl sulphate (SDS), 2-ME and 6 M urea were added before PAGE assay of toxin. These denaturing conditions were used to detect toxins of diverse molecular weights. Different molecular markers were applied to each electrophoresis: lactoalbumin (14.2 kDa), soybean trypsin inhibitor (28.5 kDa), glyceraldehyde-3-phosphate dehydrogenase (35.9 kDa), ovoalbumin (53.0 kDa), bovine serum albumin (96.4 kDa), β-galactosidase (113.6) and myosin (198.1 kDa). Calibration curves were done by plotting mobility versus log MW of molecular markers in SDS-PAGE.

Cholesterol (20 mM) suspended in 20 mM Tris-HCl pH 7.4 was sonicated and dried; 1 mL of the emulsion obtained was incubated with 10 μg of a sample of toxin containing monomer and oligomeric forms. Then, the samples were incubated with 0.1 mL Tween 80 and next 10% SDS-PAGE electrophoresis was applied to determine toxin molecular weight in presence of cholesterol and according to Ikigai et al. (1996). Coomasie Blue was applied to stain the bands of toxin and molecular markers for 60 min.

Hemolytic activity: Erythrocytes were obtained from heparinized blood of healthy individuals after University Human Ethics Committee protocol approval. Red cells were washed three times with PBS pH 6.8 before suspension preparation. The hemolytic capacity of purified toxin (315 μg/mL) was assayed in 1 mL of this buffer, incubated for 30 min at 37 °C with 0.6% of erythrocytes. The percentage of hemolysis in each case was determined by measuring the absorbance at 540 nm, and 100% of lysis was reached by incubation of erythrocytes with water. Ultrastructural alterations of erythrocytes were investigated by electronic microscopy. 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, Overkochen, Germany). Photographs were obtained by a Kodak electron imaging film.

Size of membrane alterations: Experiments were done with 1 mL of 0.6% erythrocytes incubated with of 66 kDa toxin or 13.3 kDa toxin (315 μg/mL) in presence of the following osmotic protectors: 290 mM lactose, 290 mM arabinose, 30 mM polyethylen glycol (PEG) 400, PEG 1500, PEG 4000 and PEG 6000. The hemolytic activity was measured under each osmotic condition by the liberation of hemoglobin Cauci et al. (1993).

Immunization of animals with the E.cloacae toxin: Two rabbits were injected subcutaneously with 1 mg of purified monomer of *E. cloacae* toxin every 2 weeks. Sera preimmunization was obtained, and samples of blood were bleeding between 4 and 12 weeks after immunization.

Neutralization of cytotoxic effects: Antiserum was employed in dilutions 1/10, 1/100 and 1/1000 in volume of 0.1 mL to neutralize the lytic effect of 0.1 mL of 315 μg/mL toxin, plus 0.6% human erythrocytes incubated for 30 min at 37 °C. Percentages of hemolysis were estimated respecting the 100% of lysis obtained with water by spectrophotometry at 540 nm.

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 and 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 albumin and without phenol red (1×10^6 cells/mL). Cells were stored in melting ice and used within 4 h and cell count was performed with Turks staining. HL suspensions were incubated with 50 μg/mL of monomeric or polimeric toxins for 30 min at 37 °C and cytotoxicity was assessed with Trypan Blue exclusion (Kuhnert et al., 2003). Neutralization of cytotoxic effect on leukocytes was studied by incubation of 0.1 mL dilutions of antiserum with 0.1 mL of leukocytes (10^7 cells/mL) and 0.1 mL of 4 μg/mL or 50 μg/mL toxin.

Immunoblotting of hemolysin: Monomeric and polimeric toxin forms were purified and compared by immunoblotting. Samples were subjected to SDS-PAGE with 13% polyacrylamide geles. Proteins were transferred to nitrocellulose and blocked with 0.5% Tween 20 in phosphate buffer (pH 7.2). Anti-toxin serum was employed diluted 1:1000 and incubated for 90 min. The blot was washed and treated with peroxidase-protein A and developed with 0.3% 4-chloro-1-naphtol and H_2O_2.

Inhibition of ROS stimuli with anti-toxin serum: The oxidative metabolism of neutrophils and monocytes was assayed by CL, tested by the method based on the reduction of luminol by ROS (Becerra and Albesa, 2002). The CL assay was performed in polypropylene tubes containing neutrophils or monocyte (1×10^6/mL), luminol (3.36 μM) and different concentrations of purified toxin (0.4 and 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 monocytes 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 per 10^6 cells (RUL/10^6 cells), with subtraction of the background. Control probes for cell stimulation in response to phorbol myristate acetate (PMA) stimulating agent (0.1 μg/mL) was measured by CL. The viability of leukocytes was estimated by Trypan Blue dye exclusion.

CL was applied to measure ROS generated in 0.1 ml of leukocytes suspension (10^7 cells/mL) by 0.4 μg/mL toxin previously incubated for 30 min at 37 °C with 0.1 mL of anti-toxin serum. Luminol (3.36 μM) was added to the assay and RLU were determined every 2 for 120 s. Controls with pre-immune serum were performed.

"In vivo" assay with peritoneal leukocytes: Leukotoxicity of hemolytic strain grown in blood (C_1) was compared to a non-hemolytic strain (C_4) obtained from C_1 by serial cultures without blood, until loss of hemolytic activity. Groups of six Wistar rats were inoculated in peritoneum with 1 mL of *E. cloacae* C_1 or C_4 (1×10^6 cfu/mL). After 24 h of inoculation, infiltration of leukocytes was investigated by washing the peritoneum with 1 mL HBSS to count cell death and lysis. Cell viability was continued for 5 h after extraction of leukocytes by means of Trypan Blue stain in Neubauer chamber.

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); Sephadex G-100, mercaptoethanol, lactoalbumin, tryosin inhibitor, tryosinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin, bovine albumin, alcohol dehydrogenase, trypsin, cholesterol, Ficoll-Hypaque, luminol, dextran, Trypan blue, Temed (Sigma); Bio-Rad Protein Assay, acrylamide, *N*,*N*-methylen bis acrylamide (Bio-Rad); Tris-HCL; tween 80, polyethylene glycol 400, 1500, 4000, 6000 (Anedra); Heparin (Abbott); $(NH_4)_2SO_4$, acetonitrile, trifluoracetic, sodium dodecyl sulphate (Merck); Urea, lactose, arabinose (Mallinckrodt).

Results

The strain E_2 of *E. cloacae* was selected for this work because it was the most hemolytic and leukotoxic strain. The existence of different polymeric forms was investigated by electrophoresis. SDS-PAGE without previous incubation with urea was insufficient to dissociate the polymeric forms of toxin. Urea at 6 M concentration was effective to dissociate the toxin and originate principally 13.3 kDa forms. The concentration of 13% polyacrylamide in SDS-PAGE was adequate to separate the 13.3, 27, 42 and 66 kDa and high mass toxin (Fig. 1) whereas 8% polycrylamide concentration allowed us to detect forms of toxin with 120–180 kDa, as well as small forms (data not shown).

Storage for a year of toxin increased polymeric forms. Aggregation or polymerization was evident when toxin was stored frozen for a year. The samples of toxin with different oligomeric forms increased the amount of 120 and 180 kDa polymers after storage (Fig. 2).

Assays performed with 10% SDS-PAGE also demonstrated polymerization of *E. cloacae* toxin in presence of cholesterol. Electrophoresis was useful to examine the effect of cholesterol on molecular mass of toxin, which only exhibited polymer of 66 kDa as a result of polymerization in presence of this lipid. The toxin forms of low molecular weight in runs done in presence of cholesterol were absent (Fig. 1, lane c).

During purification, hemolytic and leukotoxic activities always appeared together in the same fractions. Human erythrocytes incubated with the purified toxin (31.5 μg/mL) showed alterations such as, low hemoglobin content and swelling (Fig. 3A), but monomeric toxin to *E. cloacae* at a high

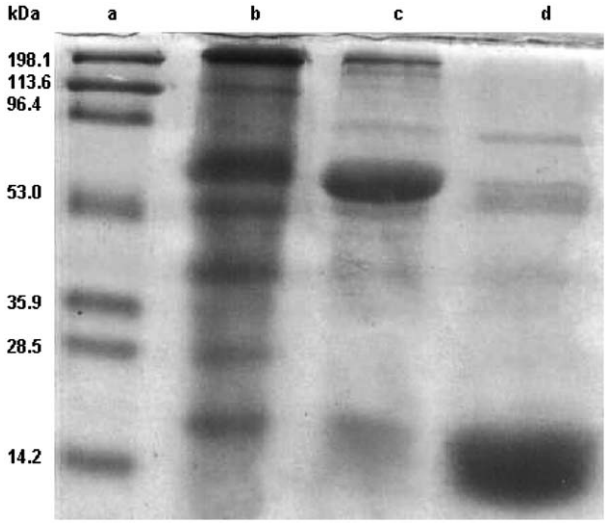

Figure 1. Oligomerization of *Enterobacter cloacae* toxin: 13% SDS-PAGE. Lane a: molecular marker. Lane b: without urea treatment, toxin sample showing different oligomeric forms (13.3, 27, 42 and 66 kDa and a high mass toxin). Lane c: polymer of toxin with molecular weight 66 kDa, formed in presence of cholesterol by incubation with toxin of "lane b". Lane d: with 6 M urea: toxin sample showing 13.3 forms.

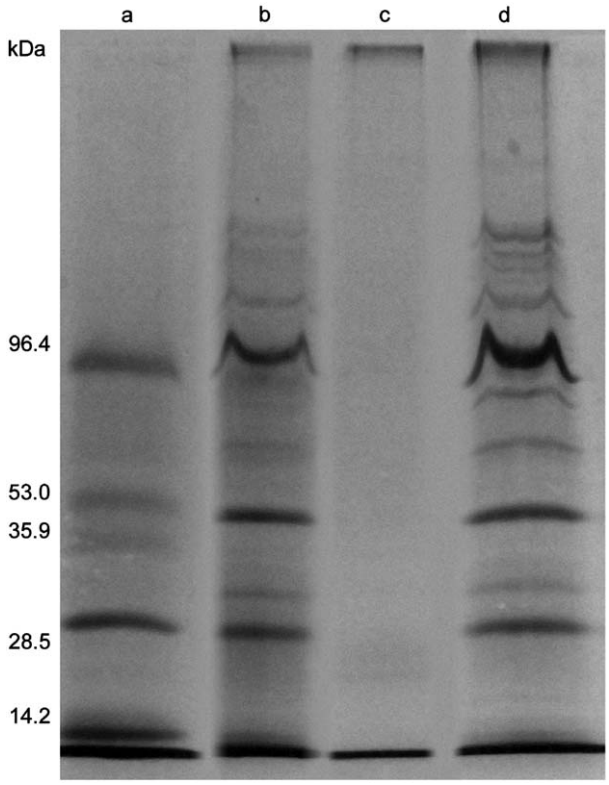

Figure 2. Effect of storage on polymerization of *Enterobacter cloecae* toxin: 10% SDS-PAGE of. Lane a: molecular markers. Lane b: polymeric toxin sample previous to storage, showing little amount of 120–180 kDa. Lane c: monomer of toxin (13.3 kDa) before storage. Lane d: the same toxin sample as "lane b" after a year of storage, showing from 120 to 180 kDa polymer increase.

concentration (315 μg/mL) had loss of hemoglobin and lysis with great amount of detritus cellular (Fig. 3B).

The size of lesions generated by *E. cloacae* toxin on erythrocyte membrane was studied by means of osmotic protective substances. Protectants of adequate size were applied to determine the functional diameter of the pores produced. Inhibition of hemolysin was obtained with PEG 6000 and PEG 4000. Molecules of smaller dimension failed to prevent hemolysis and consequent hemoglobin release. PEG 1500 induced partial inhibition of lysis and PEG 400, lactose and arabinose did not protect the erythrocytes, which released high percentages of hemoglobin (Fig. 4). The hydrodynamic diffusion diameter of osmotic protectants was assumed to be: 6.10, 3.66, 2.40, 1.26, 0.92 and 0.62 nm for PEG 6000, PEG 4000, PEG 1500, PEG 400, lactose and arabinose, respectively. The molecular diameter of functional pores formed by 13.3 kDa monomeric and 66 kDa polymeric forms were estimated in 3.66 nm.

On the other hand, rabbits developed toxin-specific antibodies, and immunization with purified toxin allowed us to obtain antiserum with a progressive increase of title. Neutralization of hemolysis was possible with samples of antiserum from 4, 8 and 12 weeks of immunization.

Immune response to *E. cloacae* toxin, through neutralizing antibodies, had the capacity to inhibit hemolytic effect (Table 1). Controls with rabbit serum before immunization failed to neutralize hemolysis and leukotoxicity.

Comparison of monomeric and polimeric toxins forms by immunoblotting revealed that both forms were reactive with specific antiserum obtained (Fig. 5).

The assays of leukotoxicity of monomeric and polimeric toxins forms showed neutrophils and monocyte lysis with 50 μg/mL. Lower toxin concentration was required to study the prelytic effect on oxidative metabolism in HL separated by Ficoll-Hypaque method. Prelytic concentration of monomer toxin (0.04–0·4 μg/mL) increased the ROS production of neutrophils at 120 s by CL. Conversely, lytic concentration of both toxin forms showed decrease in ROS, due to cell damage checked by Trypan Blue. Monocytes did not show oxidative stress at any concentrations. Both effects were neutralized by antibodies generated by toxin, oxidative stress of leukocytes produced by

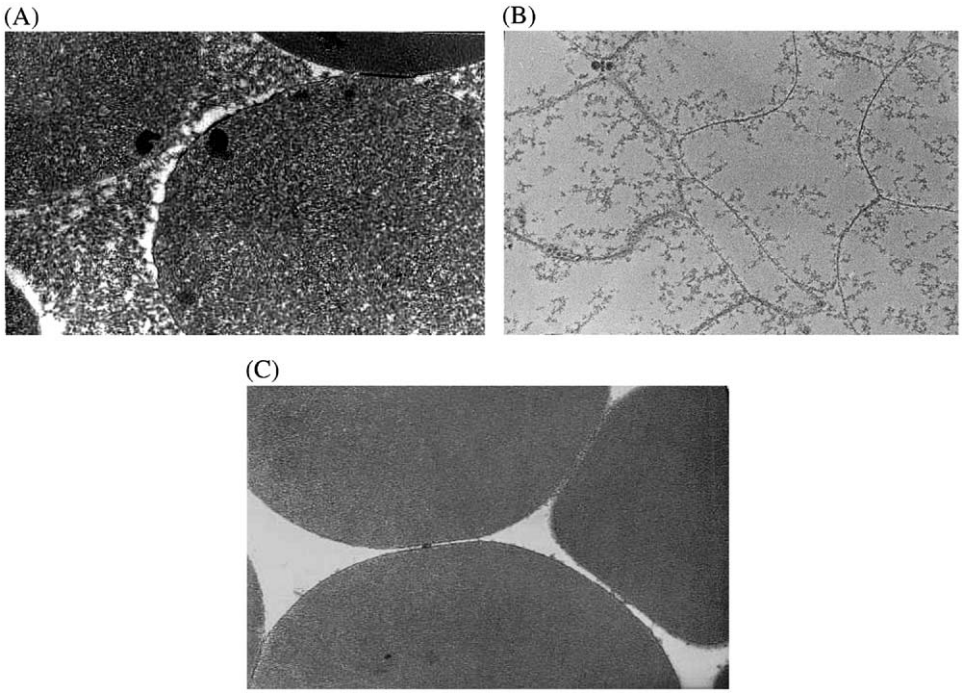

Figure 3. Electronic microscopy images of human erythrocytes exposed to monomer toxin to *Enterobacter cloecae*: (A) Human erythrocytes exposed to low concentration of toxin (31.5 μg/mL) showed a loss of hemoglobin and swelling (19000 ×). (B) Human erythrocytes incubated with monomeric toxin to *Enterobacter cloecae* at a high concentration (315 μg/mL) showing lysis with great amount of detritus cellular (12000 ×). (C) Untreated control (19000 ×)

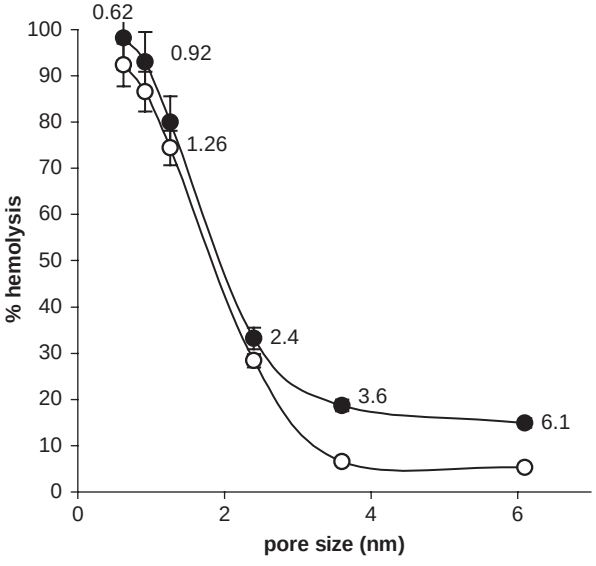

Figure 4. Pore size estimation by osmotic protection of erythrocytes. Percentages of hemolysis generated by toxin, 13.3 kDa monomer (○) and 66 kDa polymer (●), in presence of: arabinose, lactose, PEG 400, PEG 1500, PEG 4000 and PEG 6000.

monomer or polymer was antagonized by antitoxin, arriving at normal levels that generated ROS. Antiserum produced a significantly delayed of ROS

Table 1. Neutralization of hemolytic activity of *Enterobacter cloacae* toxin (315 μg/mL) by antiserum.

	Percentage of hemolysis
Without antiserum	100 ± 5.0
With antiserum of 4-week immunization	40 ± 2.1
With antiserum of 8-week immunization	32 ± 7.4
With antiserum of 12-week immunization	15 ± 2.3

($p < 0.05$) compared to samples of leukocytes incubated with toxin (Table 2).

The intraperitoneal inoculation of hemolytic (C_1) and the non-hemolytic (C_4) *E. cloacae* strain obtained from C_1 by subcultures without blood, allowed us to obtain an increase of all macrophages after 24 h. All the rats infected with C_1 and C_4 exhibited an increase of peritoneal cells over the values obtained in control by inoculation of buffer, although the macrophages of rats infected with C_1 strain evidenced high death rate of leukocytes. In C_4 treated animals, most of the leukocytes that reached peritoneum remained alive for 5 h after

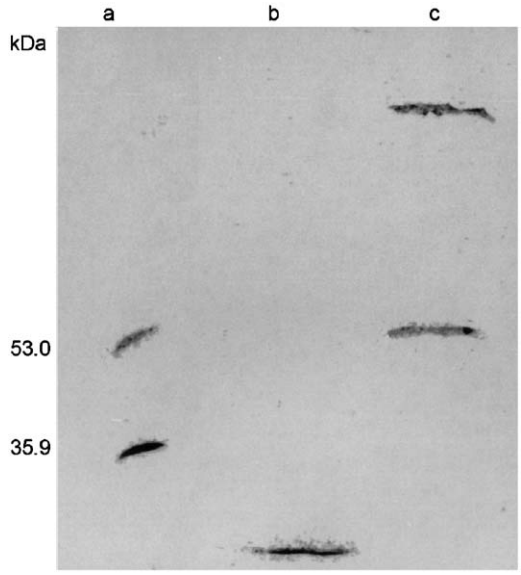

Figure 5. Immunoblotting of monomeric (Lane b) and polymeric (Lane c) *Enterobacter cloacae* haemolysin incubated with specific antiserum. Lane a: molecular markers.

Table 2. Inhibition by antiserum of reactive oxygen species stimuli generated with *Enterobacter cloecae* toxin determined by chemiluminescence with luminol at 120 s.

Samples	ROS (RUL/10^6 cells)
Leukocytes	0.23 ± 0.09
Leukocytes+monomer toxin 0.4 μg/mL	9.10 ± 0.06
Leukocytes+ monomer toxin 50 μg/mL	0.90 ± 0.07
Leukocytes+monomer toxin 0.4 μg/mL+8-week antiserum	0.92 ± 0.01
Leukocytes+monomer toxin 0.4 μg/mL+12-week antiserum	0.49 ± 0.03

extraction, indicating less cell damage than with C_1 (Table 3).

Discussion

Pore formation during the action of microbial metabolites has been described in diverse Gram-negative bacteria (Welch, 2001; Frey and Kuhnert, 2002); however, there are no reports about this property in *E. cloacae* toxin. The capacity to form oligomers of 66 kDa tetramers in the strain E_1 was evidenced by Sephadex G200 chromatography (Albesa et al., 2000), but the polymerization was best evidenced in the present study by SDS-PAGE with the new strain E_2 which produced higher amount of polymer than the strains previously investigated. The study of the new strain together with the use of 10% SDS-PAGE concentration, allowed us to demonstrate the formation of high molecular mass polymers (120 and 180 kDa) undetected with 13%. Moreover, storage of toxin frozen for a year aggregated or polymerized great amount of 120 and 189 kDa forms.

The E_2 strain exhibited higher hemolytic and leukotoxic activity than the strain E_1 initially studied (Barnes et al., 1997). Besides, this last strain showed less polymer toxin than E_2. Therefore, it was possible to explain the difference in activity of both strains taking into account that polymer exhibited higher hemolytic and leukotoxic effect than monomer, compared to previous results in our laboratory (Barnes et al., 2001a).

Polymer formed on membranes could not be separated, because the treatments to extract them from the pores immersed in membranes did not allow maintain the structure of polymers. Although, it was possible that toxin aggregated during storage in liquid medium, as a consequence

Table 3. Leukotoxic effect of *Enterobacter cloecae* toxin on rats inoculated in peritoneum with hemolytic strain (C_1) and non-hemolytic strain (C_4).

Time after extraction	Control	C_1	C_4
0 h			
Leukocytes/μL	7620 ± 163	17525 ± 8346	38950 ± 18719
% Of leukocyte death	2.7	16.6	2.6
Leukocytes/μL	7380 ± 60	6565 ± 3406	25325 ± 8343
2 h			
% Of leukocyte death	2.5	18.7	5.8
5 h			
Leukocytes/μL	5100 ± 90	2810 ± 170	24490 ± 3030
% Of leukocyte death	3.3	97.9	0.5

of its tendency to originate polymer on membranes, the folding could not be the same in aggregates than in polymeric pores.

There is evidence that supports the concept that cholesterol is an important coupling factor for cytolysins in bacteria membranes (Ziter et al., 2001). Other authors have previously showed that only cholesterol-containing liposomes allowed the assembly of *Vibrio cholerae* olygomers and their SDS-PAGE analysis indicating that monomers were converted to oligomers in presence of lipids (Ikigai et al., 1996). These antecedents are in agreement with the results obtained in the assays performed with cholesterol, which stimulated the formation of polymer of 66 kDa.

The assays with osmotic protectants suggest that monomer and polymer forms pore by oligomerization on erythrocyte membrane, showing a similar efficiency in both forms of toxins when these were incubated in the same concentration. Functional pores of monomer and polymer exhibited similar molecular diameter.

The observation of assays done indicates that polymerization, pore formation and oxidative stress are involved in the molecular events that participate in the cytotoxic effects of *E. cloacae* toxin on leukocytes. Oxidative stress seems to be the cause of leukocyte death as a consequence of infection with hemolytic strain of *E. cloacae*, according with results obtained in ''in vivo'' during the assays performed in peritoneum of rats. This toxin could be implicated in the pathogenesis of infection diseases, since leukocytes can liberate ROS by toxin action in the infiltrates causing cellular damage. Infiltration of leukocytes is a relevant phenomenon to understand the pathogenicity of bacteria able to interact with macrophages (Popoff, 1998). A common feature in infections is the infiltration of tissues by leukocytes, like as observed with *E. cloacae* in peritoneum of rats.

Inoculation of monomer toxin in rabbits induced antibodies able to neutralize hemolysis and leukotoxic effect. The neutralization of oxidative stress together with the inhibition of leukotoxic effect by means of serum anti-toxin is other evidence that oxidative stress was involved in the mechanism of action of *E. cloacae* toxin.

The immune response and the toxic-specific antiserum obtained were adequate for the purpose of this study. The neutralization of ROS stimuli and the consequent injure of leukocytes could be useful and suggest an immunoprophylatic potential of antiserum against toxin.

Finally, there are numerous intents of toxin application to obtain protection against infections (Billson et al., 1994; Menzies and Kernodle, 1996; LeClaire et al., 2002) but there are no antecedents in the toxin studied, because it is new and there exist no data about its relevance in the pathogenesis of *E. cloacae*. Consequently, it is necessary to investigate possible strategies of passive immunization to counteract the injury that this toxin can generate in leukocytes.

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