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Biological activity of *Serratia marcescens* cytotoxin

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Abstract

Serratia marcescens cytotoxin was purified to homogeneity by ion-exchange chromatography on a DEAE Sepharose Fast Flow column, followed by gel filtration chromatography on a Sephadex G100 column. The molecular mass of the cytotoxin was estimated to be about 50 kDa. Some biological properties of the cytotoxin were analyzed and compared with well-characterized toxins, such as VT1, VT2 and CNF from *Escherichia coli* and hemolysin produced by *S. marcescens*. The sensitivity of the cell lines CHO, HeLa, HEp-2, Vero, BHK-21, MA 104 and J774 to the cytotoxin was determined by the cell viability assay using neutral red. CHO and HEp-2 were highly sensitive, with massive cellular death after 1 h of treatment, followed by BHK-21, HeLa, Vero and J774 cells, while MA 104 was insensitive to the toxin. Cytotoxin induced morphological changes such as cell rounding with cytoplasmic retraction and nuclear compactation which were evident 15 min after the addition of cytotoxin. The cytotoxic assays show that 15 min of treatment with the cytotoxin induced irreversible intoxication of the cells, determined by loss of cell viability. Concentrations of 2 CD_{50} (0.56 µg/ml) of purified cytotoxin did not present any hemolytic activity, showing that the cytotoxin is distinct from *S. marcescens* hemolysin. Antisera prepared against *S. marcescens* cytotoxin did not neutralize the cytotoxic activity of VT1, VT2 or CNF toxin, indicating that these toxins do not share antigenic determinants with cytotoxin. Moreover, we did not detect gene sequences for any of these toxins in *S. marcescens* by PCR assay. These results suggest that *S. marcescens* cytotoxin is not related to any of these toxins from *E. coli*.

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- Virulence factors

Introduction

Serratia marcescens has been considered to be an important nosocomial pathogen, responsible for endemic and epidemic infections, especially in newborns and patients submitted to invasive procedures (1-4).

Studies of the virulence factors of *S. marcescens* demonstrated that clinical isolates produce a toxin with activity on Vero (African Green monkey kidney) cells in culture (5). These strains were isolated from distinct clinical sources and no relationship was observed between cytotoxic activity and strain

serotype. Moreover, this cytotoxic activity is not mediated by plasmids. The *S. marcescens* toxin is extracellular and heat labile, and optimal culture conditions were incubation at temperatures ranging from 30 to 37ºC for 24 h under shaking in medium adjusted to pH 8.5 (6).

Recently, it was shown that the hemolysin of *S. marcescens* induces cytotoxic effects on human epithelial cells, characterized by vacuolization with subsequent cell lysis (7). The cytopathic effects of the *S. marcescens* cytotoxin correspond to cell rounding followed by gradual destruction of the monolayer, as observed by inverted microscopy (5). This technique is routinely used to assess cytotoxicity in cell culture; however, it lacks sensitivity for detecting more details about early events of the cell injury process. Thus, it is still unclear whether the cytotoxin causes vacuolization and cell lysis.

We report here the purification of the *S. marcescens* cytotoxin and compare its biological characteristics with those of other well-characterized toxins.

Material and Methods

Cytotoxin purification

A 0.5-ml pre-culture of *S. marcescens* (6) was inoculated into 500 ml Davis minimal medium (8) in a 1-liter Erlenmeyer flask with shaking at 150 rpm and incubated at 37ºC for 18-24 h. Six liters of culture from 12 such flasks was centrifuged at 10,000 *g* for 15 min at 4ºC and ammonium sulfate was added slowly to the culture supernatant to 80% saturation. After centrifugation at 10,000 *g* for 15 min at 4ºC, the pellet collected was dissolved in 25 mM Tris-HCl, pH 7.0, followed by exhaustive dialysis against the same buffer. The preparation was applied to a DEAE Sepharose Fast Flow column (3.0 x 9.0 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with 25 mM Tris-HCl buffer, pH 7.0, and the cytotoxin was eluted (5-ml fractions) with the same buffer containing 0.1 M NaCl. Fractions with toxicity were pooled, concentrated to 5 ml by ultrafiltration through a PM 10 membrane (Amicon Corp., Lexington, MA, USA) and applied to a Sephadex G100 column (2.5 x 68 cm; Pharmacia) equilibrated and eluted (2.5-ml fractions) with 25 mM Tris-HCl buffer containing 0.15 M NaCl, pH 7.0. Fractions with the highest cytotoxic activity were pooled and stored at -70ºC until use. The total protein concentration was determined by the method of Bradford using reagents from BioRad (Hercules, CA, USA) and serum albumin as standard (9).

Molecular mass estimation by gel filtration chromatography

The molecular mass of the cytotoxin was estimated by gel filtration on a Superdex 200 HR column (Pharmacia). A 0.1-ml portion of purified cytotoxin was applied to the column equilibrated with 25 mM Tris-HCl buffer, pH 7.0, and eluted at 0.25 ml/min in an HPLC system (Shimadzu, Kyoto, Japan). Absorbance of the cytotoxin was measured at 280 nm and the material was collected and assayed for cytotoxicity on Chinese hamster ovary (CHO) cells. The molecular mass of the cytotoxin was estimated by comparing its elution volume with those of calibration standards such as ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa) and blue dextran 2000 (>100 kDa) (10).

Gel electrophoresis

The purified cytotoxin (30 µg/ml) was assessed for purity on 8% silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel (11).

Cell lines and cytotoxic assays

Human cervical carcinoma (HeLa), Vero,

human epidermoid carcinoma (HEp-2), baby hamster kidney (BHK-21), CHO, mouse tumor macrophage (J774) and monkey kidney (MA 104) cells, all obtained from the American Type Culture Collection (Rockville, MD, USA), were maintained in tissue culture flasks with Eagle's modified essential medium (Gibco, BRL, São Paulo, SP, Brazil) supplemented with 10% fetal calf serum, and 0.75 mM L-glutamine. The J774 cells were cultivated in RPMI medium (Gibco) under the same conditions as described above. The cytotoxicity assays were performed as described in Ref. 6. Briefly, after formation of a cell monolayer, the medium was removed from each well and replaced with fresh medium without serum and containing the cytotoxin. The plates were incubated in the presence of 5% CO_2 at 37ºC. The cell monolayer morphology was observed daily under an inverted microscope in order to detect the presence of any cytopathic effect.

Determination of cell viability and CD_{50}

In order to compare the sensitivities of the cell lines to *S. marcescens* cytotoxin, cell viability after the cytotoxic assay was quantified by the neutral red cytotoxicity assay (6). The concentration of toxin that killed 50% of CHO cells (CD_{50}) was calculated as described in Ref. 12.

Morphological changes

The CHO cells were grown on coverslips placed inside 24-well plates using 1 ml of cell suspension (1 x 10^4 cells/ml) per well and the plates were incubated at 37ºC in the presence of 5% CO_2. After 24 h, the culture medium was aspirated and replaced with 1 ml of fresh medium and a 2 CD_{50} dose of cytotoxin was added to each well. At time-defined intervals, the coverslips were washed with phosphate-buffered saline (PBS) and fixed in 10% formaldehyde solution in PBS for 1 h. The coverslips were then washed with distilled water and stained with 0.025% toluidine blue, pH 4.0, as described by Mello and Vidal (13). The coverslips were washed with distilled water, air dried, cleared in xylene and mounted on slides using Entellan (Merck, Darmstadt, Germany). The cellular alterations were observed with an Axioskop microscope (Nikon, Tokyo, Japan).

Antiserum production

Aliquots of 50 µl of a purified cytotoxin preparation (75 µg of protein per ml) were emulsified in an equal volume of Freund's complete adjuvant and injected via the inguinal node, with subsequent injections in incomplete adjuvant after 2 weeks. Animals were bled and the cytotoxicity-neutralizing capacity of the sera was tested.

Seroneutralization assays

In order to determine whether *S. marcescens* cytotoxin antiserum could neutralize the cytotoxicity of VT1, VT2 or cytotoxic necrotizing factor (CNF) produced by *E. coli*, seroneutralization assays were carried out (14). Antisera produced in rabbits against purified cytotoxin of *S. marcescens* were serially diluted in Eagle's medium and mixed with 1/40 dilution of the test toxins. The mixtures were incubated for 1 h at 37ºC, applied to Vero cells and incubated for 72 h at 37ºC. The plates were examined microscopically on a daily basis for the appearance of cytopathogenicity. Negative controls with preimmune serum were also included.

Hemolysis assays

The hemolytic activity of the purified cytotoxin was assayed as previously described (7). A 2 CD_{50} dose (0.56 µg/ml) of cytotoxin was serially diluted (base 2) in 0.9% NaCl and 100-µl aliquots of each dilu-

tion were mixed with 1 ml of erythrocyte suspension and incubated for 30 min at 30ºC. The mixture was then centrifuged for 3 min at 2,500 rpm to remove unlysed erythrocytes and cell debris. The absorbance of the supernatants containing released hemoglobin was measured at 405 nm. Results were compared with total lysis (100%) caused by SDS used as control.

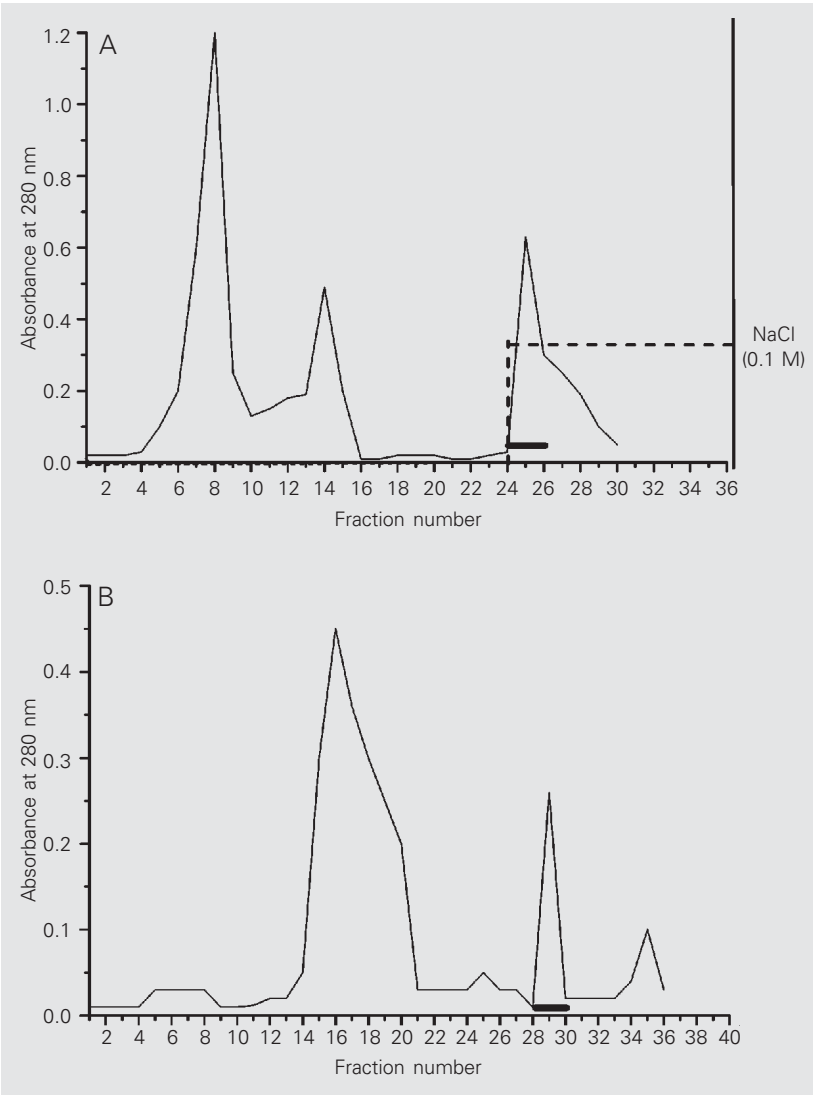

Figure 1. *A*, DEAE Sepharose Fast Flow column chromatography. A crude sample was applied to a DEAE Sepharose Fast Flow column equilibrated with 25 mM Tris-HCl buffer, pH 7.0. Fractions with cytotoxic activity (5 ml) were eluted with a nonlinear gradient of 0.1 M NaCl in the same buffer. The horizontal bar indicates the fractions pooled for subsequent purification. *B*, Sephadex G100 column chromatography. The cytotoxic fractions (5 ml) obtained from the DEAE Sepharose column were applied to a Sephadex G100 column equilibrated with 25 mM Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl. The horizontal bar indicates the fractions with cytotoxic activity.

Toxin gene sequence determination by genotypic analysis (PCR)

The *S. marcescens* strain was screened for the presence of gene sequences of toxins from *E. coli* using primers for VT1 (ST-I), VT2 (ST-II) (15) and CNF (16). Bacterial DNA to be amplified was released from the organism by boiling. The reactions were performed as previously described (17) using 2 µl dNTP solution containing 20 mM of each nucleotide, 1 µl Taq DNA polymerase (1 U/µl), 3 µl 5 µM $MgCl_2$, 5 µl PCR buffer, 5 µl DNA, 1 µl of each primer (50 pmol), and 35 µl sterile Milli-Q water. The solutions were pre-heated without the enzyme at 94ºC/10 min and submitted to 30 cycles (GeneAmp PCR System 9700, Perkin Elmer, Foster City, CA, USA) of 94ºC/2 min, 50ºC/2 min and 72ºC/1 min (VT2), 94ºC/2 min, 57ºC/2 min and 72ºC/1 min (ST-I and CNF); after these cycles they were submitted to 72ºC/7 min. PCR products were analyzed by 2% horizontal agarose gel electrophoresis with ethidium bromide staining under UV light.

Results

Cytotoxin purification

The crude cytotoxin preparations were initially subjected to ion-exchange chromatography on a DEAE Sepharose Fast Flow column. The column was eluted with 500 ml of 25 mM Tris-HCl buffer at a flow rate of 1 ml/min, and the fractions containing cytotoxic activity were eluted with the same buffer containing 0.1 M NaCl (Figure 1A, horizontal bar). The cytotoxin was subsequently chromatographed on a Sephadex G100 gel filtration column equilibrated with 25 mM Tris-HCl buffer containing 0.15 M NaCl. Cytotoxic activity was eluted in the third peak (Figure 1B, horizontal bar). Cytotoxin obtained at this stage presented a single

protein band by SDS electrophoresis (Figure 2). The cytotoxin peak was eluted between the positions for bovine serum albumin and ovalbumin, corresponding to an apparent molecular mass of 47 to 50 kDa on the calibration curve.

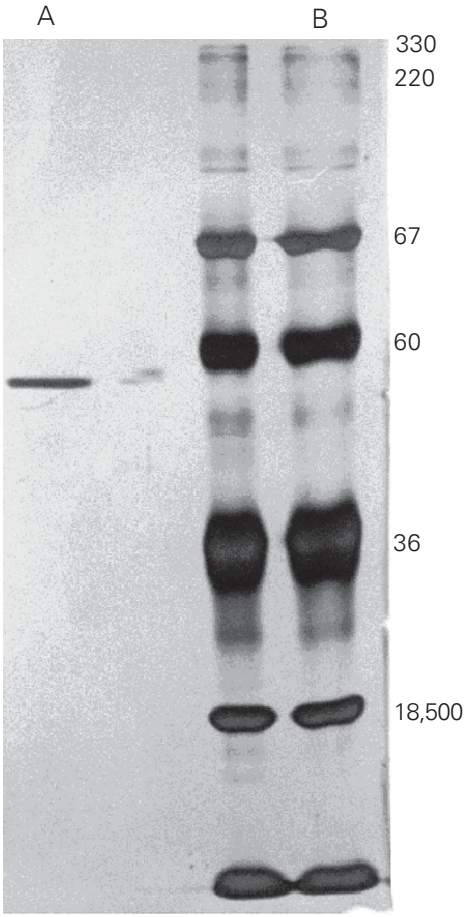

Figure 2. Analysis of *Serratia marcescens* cytotoxin by SDS-PAGE using silver staining. *Lane A*, Cytotoxic fraction obtained from a Sephadex G100 column (30 µg/ml); *lane B*, molecular weight markers.

Cytotoxicity and cell viability assays

Among the cell lines tested, CHO, HeLa, HEp-2, Vero, BHK-21 and J774 were susceptible to *S. marcescens* cytotoxin and only MA 104 cells were resistant. Although the susceptible cell lines showed similar morphological changes when exposed to cytotoxin, the cells were not affected to the same extent after exposure for a specific time. The highest sensitivity was observed with CHO and HEp-2 cells, with the cytopathic effect starting as early as 15 min after incubation with toxin and resulting in the destruction of the monolayer within 1-2 h (Figure 3). The BHK-21, HeLa, J774 and Vero cells showed monolayer destruction after 24 h, as determined by the viability assays. Thus, the CHO cells were used in all experiments for the characterization of the cytotoxin. The CD_{50} of the cytotoxin is defined as the highest concentration of the sample that killed 50% of the CHO cells after 24 h. The purified cytotoxin had a CD_{50} of 0.28 µg/ml.

Morphological changes

Morphological changes induced by the cytotoxin were studied in CHO cells stained with toluidine blue after different incubation periods. The first observable changes were evident about 15 min after the addition of the cytotoxin. The cytoplasm started to retract and the cells became rounded, with progressive nuclear compactation and extensive surface blebbing being observed after 1 h (Figure 4B). The morphological changes induced by the cytotoxin were then compared with *S. marcescens* hemolysin and VT1, VT2 and CNF from *E. coli* (Table 1).

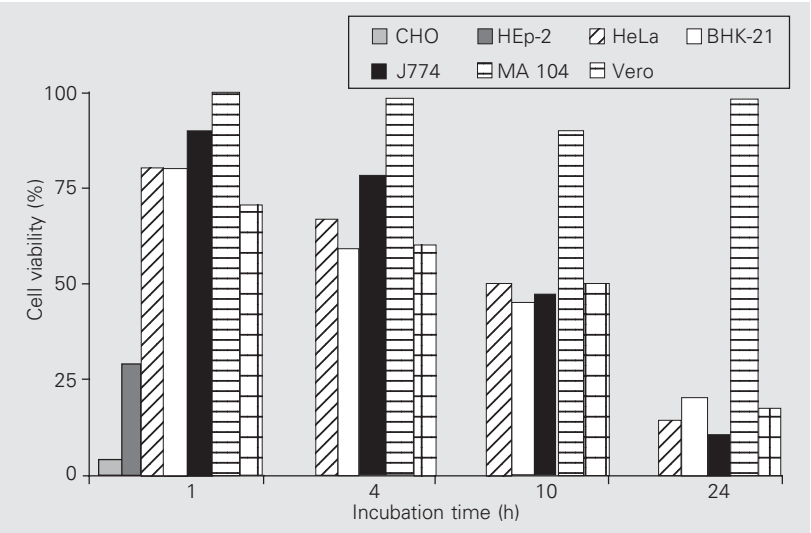

Figure 3. Response of the CHO, HEp-2, HeLa, BHK-21, Vero, MA 104 and J774 cell lines after different incubation periods with a 2 CD_{50} dose (0.56 µg/ml) of purified *Serratia marcescens* cytotoxin. Cell viability was measured by neutral red assay.

Hemolysis assays

S. marcescens cytotoxin did not show hemolytic activity in the liquid hemolytic assay, even when treated with crude cytotoxin preparations (2 CD_{50} dose or 0.56 µg/ml).

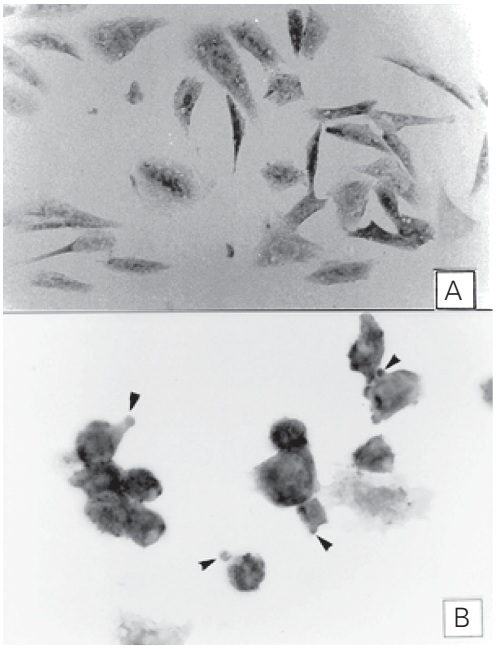

Figure 4. Morphological changes induced by purified *Serratia marcescens* cytotoxin (2 CD_{50} dose) in CHO cells stained with toluidin blue (100X). *A*, Typical morphology of the control cells; *B*, cytoplasmic retraction, nuclear compactation and cytoplasmic blebbing (arrowheads) after 30 min of treatment.

Serum neutralization assays

At higher concentrations of antitoxin serum, i.e., lower dilution, the morphological changes induced by VT1, VT2 and CNF toxins from *E. coli* on Vero cells were clearly evident after 24 h. The results of neutralization studies showed that antiserum against *S. marcescens* cytotoxin did not neutralize the biological activity of these toxins (Figure 5).

Genotypic analysis (PCR)

The assays showed that *S. marcescens* isolates do not amplify any detectable fragment, being characterized as negative for amplification by PCR gene sequence for VT1, VT2 and CNF toxins.

Discussion

The *S. marcescens* cytotoxin used in the present study was purified to homogeneity from a culture supernatant grown in Davis minimal medium (8) by ion-exchange chromatography on a DEAE Sepharose Fast Flow column and submitted to gel filtration on a

Table 1. Characteristics of toxins that were compared with *Serratia marcescens* cytotoxin in this study.

Bacterial toxin	Type of effect	Sensitive cell lines*	Time of cytopathic effect**	Reference
S. marcescens cytotoxin	Cell rounding, cytoplasmic retraction	CHO, HEp-2, HeLa	15 min	Present study
S. marcescens hemolysin	Vacuolization, cell lysis	Erythrocytes, HeLa, HEp-2	10 min	7
E. coli VT1	Round cells with a shriveled appearance	Vero, HeLa	24 h	19
E. coli VT2	Round cells with a shriveled appearance	Vero, HeLa	24 h	19
E. coli CNF	Formation of giant cells, multinucleation	HeLa, Vero	24 h	19

*The most common cells used for the cytotoxicity assays.
**Approximate time for the appearance of a cytopathic effect after toxin addition.

Sephadex G100 column. The fractions with cytotoxic activity were eluted in the third peak (Figure 1B, horizontal bar) and revealed a single protein band by SDS electrophoresis (Figure 2). The molecular mass of the purified cytotoxin was estimated to be 47 to 50 kDa by gel filtration chromatography. This method is based on a calibration curve using proteins of known molecular masses as markers (10). The results show that the molecular mass of cytotoxin is apparently different from extracellular proteases produced by this bacterium, which have molecular masses of 56, 60 and 73 kDa (18). However, this cytotoxin has not yet been characterized in terms of proteolytic activity.

In order to choose a suitable target cell line, we compared the sensitivity of several cell lines to cytotoxin produced by clinical isolates of *S. marcescens*. This represents an important step related to the practical aspects of characterization of the cytotoxin. CHO and HEp-2 cells appeared to be the most sensitive to the toxic effects of *S. marcescens* cytotoxin, followed by BHK-21, HeLa, J774 and Vero cells (Figure 3), while MA 104 cells were resistant to the toxin. Thus, CHO cells were used as a model to study the details of the mechanism of action of the cytotoxin.

The pattern of morphological changes gives a preliminary idea of the characteristics of the toxin and its mechanism of action

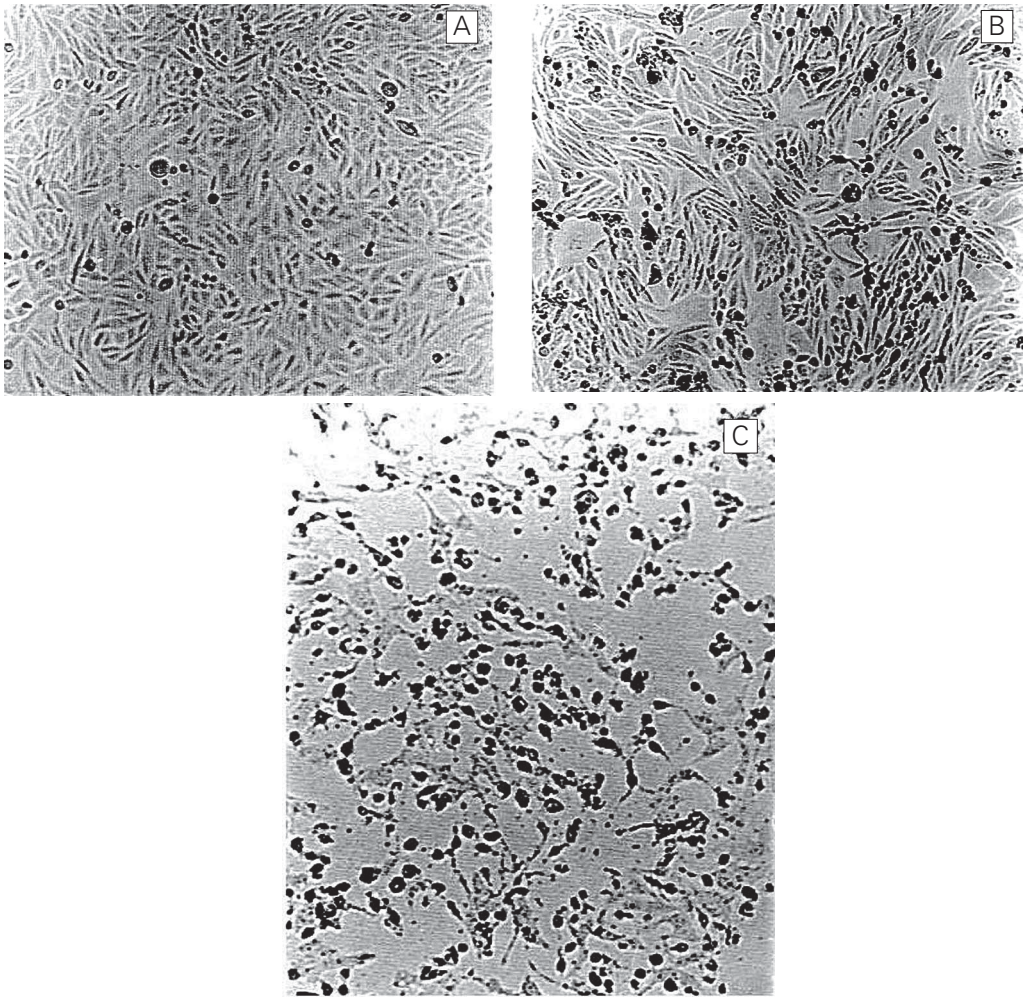

Figure 5. Effects of VT1 and CNF toxins on Vero cells in seroneutralization assays. *A*, Vero control cells. *B*, Vero cells that received mixtures of antiserum against *Serratia marcescens* cytotoxin incubated for 1 h at 37ºC with CNF toxin. *C*, Vero cells inoculated with mixtures of antiserum and VT toxin.

on the cells. The cells treated with *S. marcescens* cytotoxin lost the typical morphology observed in the untreated cultures (Figure 4A) and showed cell rounding, cytoplasmic retraction, compactation of the nuclei and cytoplasmic blebbing (Figure 4B). The morphological changes induced in cell lines and the time of first observable effects are useful to distinguish the *S. marcescens* cytotoxin from other bacterial toxins, as shown in Table 1. The cell rounding seen in CHO cells in response to the *S. marcescens* cytotoxin is different from the cell rounding with shriveled appearance induced by VT1 and VT2 or from the giant, multinucleated cells induced by CNF from *E. coli* (19). The lack of neutralization of cytotoxic activity of VT1, VT2 and CNF by antisera prepared against *S. marcescens* cytotoxin indicates that these toxins do not possess an antigenic relationship. Moreover, cytotoxin-producing *S. marcescens* was negative when tested for the presence of gene sequences for VT1, VT2 and CNF toxins. The results of all comparative studies showed that cytotoxin is not related to any of the toxins studied.

Almost all strains of *S. marcescens* secrete a hemolysin that lyses all mammalian erythrocytes tested and is cytotoxic to human epithelial cells. The cytopathic effects of hemolysin on HEp-2 cells were characterized by rapid vacuolization (15 min) followed by lysis after 40 min (7). These morphological changes observed in cultured cells were clearly distinct from those of cytotoxin, as shown in Table 1. Therefore, the purified cytotoxin did not cause hemolytic activity in liquid assays. This is of particular importance since this study provides clear evidence that the cytotoxin is distinct from *S. marcescens* hemolysin.

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