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Cytotoxic Activity of *Serratia marcescens* Clinical Isolates

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Abstract

Twenty *Serratia marcescens* isolates from clinical specimens were examined for their cytotoxic activity on four cell lines (HEp-2, Vero, CHO, J774). Most of the isolates were found to be cytotoxic to CHO (70%), Vero (75%) and HEp-2 cells (90%). CHO cells were the most sensitive to cell-free supernatants, followed by HEp-2 and Vero cells. Two strains produced cytotonic toxins which caused elongation of CHO cells. Moreover, twelve isolates (60%) revealed cytotoxic potential to macrophage cell line J774. The results indicate that these bacteria may destroy phagocytes and epithelial cells, which may lead to spread within the host.

Key words: *Serratia marcescens*, cytotoxicity

Introduction

Strains of *Serratia marcescens* have been recognized as an important nosocomial pathogen causing a variety of diseases, including respiratory and urinary tract infections, meningitis, peritonitis and bacteremia. The bacteria are becoming increasingly important cause of many outbreaks and endemic nosocomial infections, particularly among newborns and patients submitted to invasive procedures (Buffet-Batoillon *et al*., 2009; Friedman *et al*., 2008; Grimont and Grimont, 2006). Although *S. marcescens* is the common *Serratia* species causing nosocomial infections, little is known about the factors impacting their pathogenicity and virulence. The possible mechanism of the pathogenesis is complex and multifactorial, with the involvement of a number of putative virulence factors whose role in development of disease is not clear. The first step of pathogenesis is colonization of epithelial cells. After adhesion to the cells strains produce many potential virulence factors, including extracellular toxins which are probably the most common mechanism of pathogenicity. Some *S. marcescens* isolates produce hemolysin, which represents the prototype of a family of pore forming toxins with hemolytic and cytotoxic activity (Hertle, 2005). Strains of *S. marcescens* have been reported to produce many extracellular proteins, including proteinases, lipases, nucleases, chitinases, lecithinases, which may directly contribute to cellular cytotoxicity by exerting their damaging effects upon host cells (Grimont and Grimont, 2006).

The major defense mechanism of host nonspecific immunity represents cell-mediated killing. Phagocytic cells such as macrophages and neutrophils contribute to the primary line of innate defense against bacterial pathogens by providing their removal and destruction at the level of the epithelial barrier. Therefore, many bacterial pathogens have developed specific strategies to suppress the effective antimicrobial immune response of macrophages to avoid the innate immune defense of the host (Navarre and Zychlinsky, 2000).

S. marcescens may infect numerous sites of the host body, including lungs and respiratory epithelia, muscle and soft tissues, therefore in the current study, we have examined the cytotoxic activity of human isolates to different mammalian epithelial cell lines (Vero, CHO, HEp-2). Moreover, we investigated the effect of *S. marcescens* cell-free filtrates on macrophage cell line J774.

Experimental

Materials and Methods

Bacterial strains. A total of 20 isolates (listed in Table I) identified as *Serratia marcescens* by biochemical test kit (API20E, bioMerieux) were analyzed. They were originated from specimens of hospitalized

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Table I
Serratia marcescens strains used in the study

Source of origin (number of strains)	Isolates No
Ulceration (4)	MPU S1, MPU S4, MPU S7, MPU S23
Urine (4)	MPU S3, MPU S12, MPU S18, MPU S21
Postoperative wound (3)	MPU S6, MPU S9, MPU S11
Catheter (1)	MPU S20
Feces (1)	MPU S15
Blood (1)	MPU S22
Aspirate (2)	MPU S2, MPU S10,
Pus: pharynx, ear, abscess, drain (4)	MPU S5, MPU S14, MPU S19, MPU S13

patients and belonged to Bacterial Culture Collection of Department of Microbiology Poznań University (MPU). Seven isolates originated from wounds (postoperative and ulcerations), 4 from secretions (2 from aspirates and 1 from ear and pharynx), 4 from urine, 1 from blood, 2 from catheter and drain, 1 from fecal specimen. The isolates were maintained at –75°C in trypticase soy broth (TSB, Difco) containing 50% (vol/vol) glycerol.

Cell cultures. Human epidermoid carcinoma cells from the larynx (HEp-2), Chinese hamster ovary cells (CHO) and African monkey kidney (Vero) were cultured in Eagle Minimum Essential Medium (EMEM, Sigma) with 5% fetal calf serum (FCS, Sigma) containing 2 mM glutamine, 50 IU of penicillin per milliliter, streptomycin (100 µg/ml) and nystatin (1 mg/ml). The murine macrophage cell line, J774 was maintained in growth medium (GM), containing RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, gentamicin (5 µg/ml) and 2 mM L-glutamine (Krzymińska *et al*., 2009) The cells in number 1×10^4 cells per well seeded with 100 ml of suspension and incubated at 37°C in an atmosphere with 5% CO_2.

Preparation of bacterial cell-free supernatants. For preparing bacterial filtrates, the strains were cultured in brain heart infusion broth (BHI, Difco) at 37°C. The cultures were incubated on Luria-Bertani medium (LB, Difco) in a shaking incubator with agitation at 300 rpm at 37°C for 24 h. After centrifugation at 3000 rpm for 20 min, the supernatants were sterilized through 0.22 mm-pore size membrane filters Millex-GV (Millipore). Sterile culture supernatants were heated at 56°C for 20 min to destroy activity of heat-labile toxins (Carbonell *et al.*, 1997).

Cytotoxic and cytotonic activity to epithelial cells. The assay was performed according to Krzymińska *et al*. (2009). Twofold serial dilutions (from 1:2 to 1:512) of culture filtrates in phosphate buffered saline (PBS, Biomed) added to the wells of tissue culture plate containing confluent Vero and CHO monolayers and incubated for 24 hours at 37°C. As negative controls the wells received non-pathogenic *E. coli* K12C600 filtrates. Cytotonic activity revealed as elongation of CHO cells. The cytotoxic titer of each isolate was calculated by determining the reciprocal of the highest dilution of culture filtrates which produced a cytopathic effect. The results were observed under an inverted microscope. The results were presented as mean titers from two experiments in triplicate.

Cytotoxic activity to murine macrophage J774 cells by Neutral Red retention assay. Neutral Red (NR) is a biomarker of cellular stress and supravital dye taken up in the lysosomes of viable cells (Maleri *et al.*, 2008). The assay was performed in microtitration plates with a method by Carbonell *et al.* (1997) with slightly modifications. Macrophage monolayer was incubated with bacterial culture filtrates at 37°C for 24 h. As a negative control, the cells were infected with non-pathogenic *E. coli* K12C600 filtrate. Next, the medium was removed and the cultures were washed with PBS and 200 µl of NR (50 µg/ml) was added to each well and incubated for 3 h at 37°C. After incubation the dye solution was aspirated, cells were rinsed in PBS before being fixed with formalin in calcium chloride solution (40% formaldehyde, 10% anhydrous calcium chloride) which was next removed, and incorporated dye was eluted from the cells by adding ethanol/acetic acid mixture (50% ethanol and 1% acetic acid). The absorbance at 540 nm was measured using a plate reader. All absorbance values were corrected against blank wells which contained growth medium alone which were processed as described above. Cell viability was determined by comparing the absorbance values obtained from the control wells (taken as 100% viability).

Results

Cytotoxic and cytotonic activity to epithelial cells. Cytotoxic potential of *S. marcescens* isolates was studied on three different cell lines, including Vero, CHO and HEp-2 cells. Microscopic examination of the cells following incubation with cell-free supernatants revealed a number of changes: rounding and shrinking of cells, followed by detachment, loss of cytoplasmic extension, disorganization of cell monolayer (Fig. 1B, E, G.). Cytotoxicity to CHO cells (Fig. 1B) was induced by 14 strains (70%) with cytotoxic titers ranging from 1 to 128. Eighteen of 20 (90%) *S. marcescens* strains were found to be cytotoxic to HEp-2 cells (Fig. 1E) with cytotoxic titers ranging from 1 to 32. The highest cytotoxic activity revealed 10 isolates (from MPU S1 to MPU S7, MPU S10, MPU S11, MPU S22). Fifteen strains (75%) were cytotoxic to Vero cells (Fig. 1G) with lower cytotoxic titer

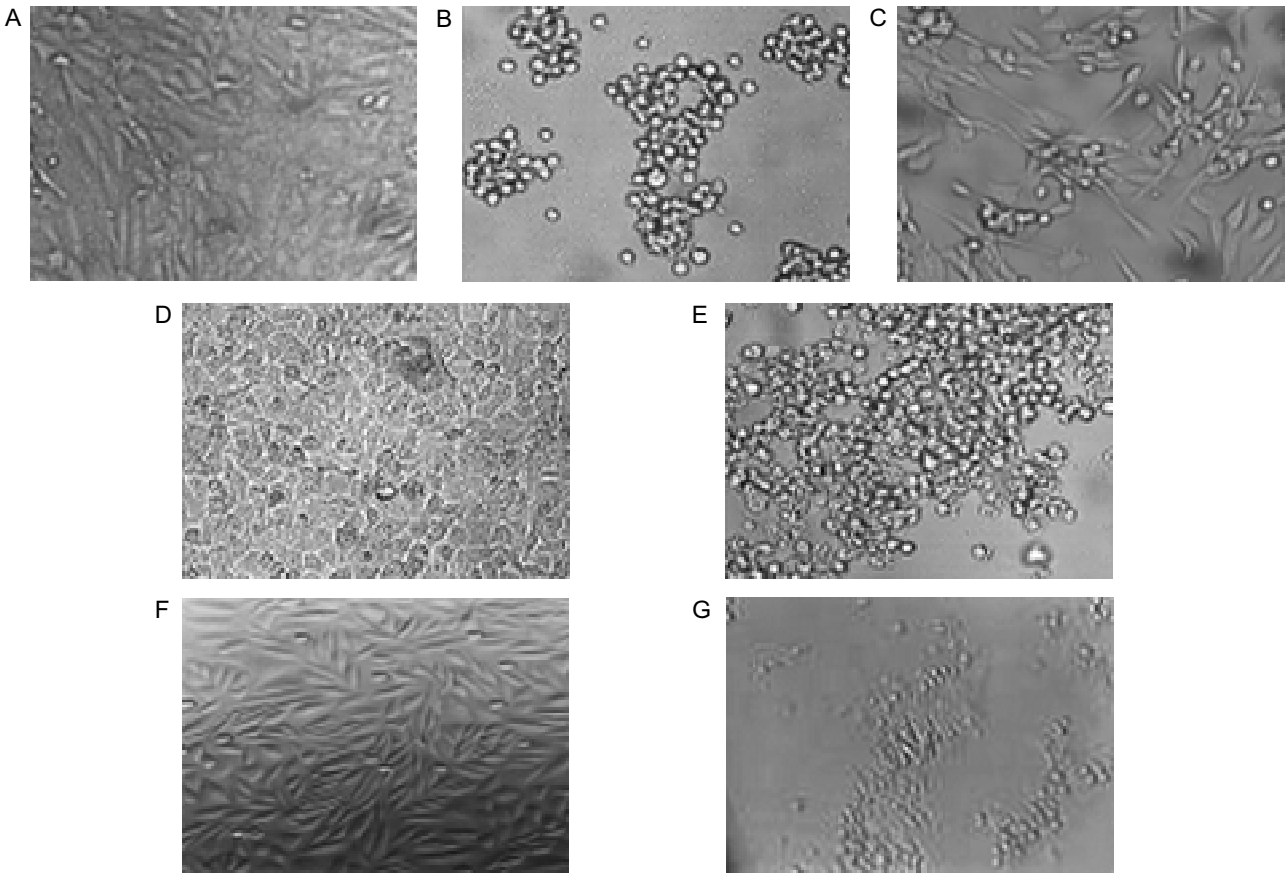

Fig. 1. Cytotoxic effects of *S. marcescens* culture filtrates to CHO (B), HEp-2 (E) and Vero cells (G). Cytotonic activity of *S. marcescens* MPU S15 to CHO cells (C). A, D, F – uninfected cells. Magnification, ×100.

(1–32). Preheating (56°C for 20 min) of the supernatants caused a decrease in cytotoxic activity to CHO, Vero and HEp-2 cells (Table II). Mean cytotoxicity titers for individual cell lines taking all isolates into account were in the 3.55–22.7 range (Table III). CHO cells expressed the highest sensitivity with mean titer 22.7±0.11 whereas the least sensitive were Vero cells (mean titer = 3.6±0.07). Cytotonic activity identified as elongation of CHO cells (Fig. 1C) was observed for 2 isolates (MPU S3, MPU S15). Non pathogenic strain *E. coli* K-12 C200 was not cytotoxic to epithelial cells.

Cytotoxic activity of *S. marcescens* strains to murine macrophages. Twelve strains (60%) were cytotoxic to murine J774 cells after 24-hour infection, as measured by NR assay (Table IV). The maximum cytotoxicity (52–68%) was observed in macrophages infected with two strains (MPU S21, MPU S22), the lowest cytotoxic activity was demonstrated by seven strains (35%). Cytotoxic activity to J774 cells was not inhibited after preheating of the supernatants. *E. coli* K-12 C200 strain, the negative control, was not cytotoxic to murine macrophages.

Table II
Cytotoxic activity of culture supernatants of *S. marcescens* strains

Mean titers[1]	CHO		Vero		HEp-2	
	Non treated supernatant	Heat inactivated supernatant	Non treated supernatant	Heat inactivated supernatant	Non treated supernatant	Heat inactivated supernatant
64 – 128	3 (15)[2]	0	0	0	0	0
8 – 32	6 (30)	1 (5)	2 (10)	1 (5)	9 (45)	2 (10)
1 – 4	5 (25)	7 (35)	13 (65)	8 (40)	9 (45)	10 (50)
0	6 (30)	12 (60)	5 (25)	11 (55)	2 (10)	8 (40)

[1] Mean of the reciprocal of the highest dilution yielding rounding, detachment and destruction of 50% of CHO, Vero or HEp-2 cells.
[2] Number (and percentage) of strains that revealed cytotoxic activity.

Table III
Mean and standard error (SE) of cytotoxicity titers for different cell lines

Mean ± SE	Cell line
CHO	22.7 ± 0.11
HEp-2	10.8 ± 0.08
Vero	3.6 ± 0.07

Table IV
Cytotoxic activity of *S. marcescens* culture supernatants to murine J774 macrophages

Cytototoxicity range[1]	Number of cytotoxic isolates (%)	
	Non treated supernatant	Heat inactivated supernatant
0	8 (40)[2]	9 (45)
7.8 – 19.2	7 (35)	7 (35)
21.6 – 29.4	3 (15)	2 (10)
52.3 – 68.1	2 (10)	2 (10)

[1] The percentage of cytotoxicity was determined 24 h after infection by NR assay,
[2] Number and (percentage) of strains revealing cytotoxic activity.

Discussion

Serratia marcescens is increasingly recognized as a cause of morbidity in nosocomial units. It has been considered as an etiologic agent in all kinds of infection in humans. However, the exact mechanism of pathogenicity has not been sufficiently understood.

The analysis of incidence of cytotoxic activity in *S. marcescens* isolates revealed that most strains produced cytotoxic toxins, which were noticed in the case of CHO (70%), Vero (75%) and HEp-2 cells (90%). The highest cytotoxic activity was detected in strains isolated from ulceration (3), postoperative wounds (2) and one each from urine, blood, aspirate and pus. Previously, Carbonell *et al*. (1997) demonstrated that culture filtrates from 22% *S. marcescens* isolates were cytotoxic to Vero and HeLa cells. Marty *et al*. (2002) confirmed earlier reports that the strains are cytotoxic to mammalian cells. Toxin production by *S. marcescens* strains is still not clearly defined. Marty *et al*. (2002) examined the 56-kDa metalloprotease of *S. marcescens* strains and found it to be the most potent cytotoxic factor. They suggested that the enzyme may possess a binding site for specific host proteins that are internalized by an endocytic mechanism into host cells. Carbonell *et al*. (2004) isolated a cytotoxic enterotoxin from a clinical isolate of *S. marcescens* which was highly cytotoxic to CHO cells but did not reveal hemolytic activity, suggesting that the cytotoxin is distinct from *S. marcescens* hemolysins. In consecutive reports, Carbonell *et al*. (2003) observed that the cytotoxic toxin was bound to the CHO cell surface, without being internalized and was able to trigger changes in the intracellular metabolism of the cells and to induce cell death by apoptosis. In the past several years there have been reports about the family of *Serratia*-type pore forming toxins (Hertle, 2005). The hemolysin (ShlA) represents the prototype of a new type of cytolysins which are distinct from *E. coli* type α-hemolysins, staphylococcal α-toxins or other related toxins. ShlA pore formation in nucleated eukaryotic cells and erythrocytes results in cell lysis. The toxin additionally brings about cytoskeleton rearrangement and apoptosis.

The *S. marcescens* culture filtrates incubated at 56°C for 20 min revealed a decrease in cytotoxic activity to CHO (86% filtrates), HEp-2 (90% filtrates) and to Vero cells (43%). The strains probably produce heat-labile cytotoxins. Carbonell *et al*. (2003) showed that a monolayer of Vero cells lost 30% cell viability when the filtrates were heated to 60°C, and no cytopathic effect was observed after incubation at 70°C.

In order to choose a suitable target cell line, we compared the sensitivity of three epithelial cells to the cytotoxic activity of clinical isolates. In the present study CHO cells appeared to be the most sensitive to the toxic effect of *S. marcescens* culture filtrates, followed by HEp-2 and Vero cells. The results indicate that CHO cells could be used as a model to study the exact mechanism of action of cytotoxic factors. Carbonell *et al*. (2003) also found that CHO and HEp-2 cells are highly sensitive to *S. marcescens* cytotoxin.

Interestingly, two isolates – MPU S3 and MPU S15, appeared to be positive for cytotonic activity, which revealed as elongation of CHO cells. Singh *et al*. (1997) noticed that 4 of 6 *S. marcescens* strains isolated from food produced cytotonic toxins. There is no evidence of cytotonic toxins production by strains originating from human specimens. Cytotonic and heat-labile enterotoxins were produced, respectively, by *Vibrio cholerae* and *E. coli* strains. The toxins activated adenylate cyclase, which caused an increase in intracellular cAMP concentration, inducing morphological alterations in CHO cells and producing an imbalance in electrolyte movement in epithelial cells, resulting in abundant net fluid loss from the intestine (Sanchez *et al*., 2005). The high cAMP concentration impairs host cells functions, such as phagocytosis and oxidative ability (Moss *et al*., 2000). Moss *et al*. (2000) reported that *Bordetella pertusis* produced an adenylate cyclase-hemolysin (AcHly) toxin which caused increased cAMP level. An increase in the intracellular concentration of cAMP leads to apoptosis of mammalian cells.

Phagocytes, either resident in tissues or circulating in blood contribute to the primary line of innate defense against bacterial pathogens by providing their removal and destruction at epithelial barrier level (Navarre and Zychlinsky, 2000). Some bacterial ente-

ropathogens have developed strategies for avoiding antimicrobial effects of phagocytes and have evolved mechanisms which kill the immune cells. In this study we have demonstrated that 60% of isolates were cytotoxic to murine macrophages J774 cells. The highest activity was observed for strains isolated from blood and urine. The results suggest that the cytotoxic activity of these bacteria may be an important mechanism for evasion of host immune response during infection, which may induce inflammation and development of fatal bacteremia in weakened patients.

The results of the study demonstrate that *S. marcescens* clinical isolates reveal cytotoxic activity that may modulate the properties of host epithelial cells. Moreover, we have observed the incidence of an antihost strategy based on the elimination of host immune cells.

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