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Cytotoxicity of *Vibrio vulnificus* Cytolysin on Rat Neutrophils

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Cytolysin produced by *Vibrio vulnificus* has been known to be lethal to mice by increasing vascular permeability and neutrophil sequestration in the lung. In the present study, a cytotoxic mechanism of *V. vulnificus* cytolysin on the neutrophil was investigated. Cytolysin rapidly bound to neutrophils and induced cell death, as determined by the trypan blue exclusion test. *V. vulnificus* cytolysin caused the depletion of cellular ATP without the release of ATP or lactate dehydrogenase. Formation of transmembrane pores was evidenced by the rapid efflux of potassium and 2-deoxy-D-[^{3}H]glucose from cytolysin-treated neutrophils. It was further confirmed by the rapid flow of monovalent ions in the patch clamp of cytolysin-treated neutrophil membrane. The pore formation was accompanied by the oligomerization of cytolysin monomers on the neutrophil membrane as demonstrated by immunoblot, which exhibited a 210 kDa band corresponding to a tetramer of the native cytolysin of M_r 51,000. These findings indicate that *V. vulnificus* cytolysin rapidly binds to the neutrophil membrane and oligomerizes to form small transmembrane pores, which induce the efflux of potassium and the depletion of cellular ATP leading to cell death without cytolysis.

Keywords: Cytolysin, Neutrophil, *Vibrio vulnificus*.

Introduction

Vibrio vulnificus is an estuarine bacterium that causes serious septicemia and wound infection in humans (Hollis *et al.*, 1976; Blake *et al.*, 1979). Persons who are immunocompromised or have underlying diseases such as liver cirrhosis are more vulnerable to *V. vulnificus* infection, and the mortality rate approaches 70% in primary septicemia (Park *et al.*, 1991). However, the pathogenic mechanism of this infection is not yet clear . A variety of factors, including an extracellular cytolysin (Gray and Kreger, 1985), an elastolytic protease (Miyoshi *et al.*, 1987), the presence of a polysaccharide capsule (Kreger *et al.*, 1981), resistance to bactericidal effects of sera (Johnson *et al.*, 1984), resistance to phagocytes (Yoshida *et al.*, 1985), and the ability to acquire iron from transferrin (Bullen *et al.*, 1991) have all been implicated as possible virulence determinants for *V. vulnificus* in animal models. Kreger and Lockwood (1981) demonstrated that cytolysin present in the culture medium of *V. vulnificus* showed hemolytic and lethal activity and acted as a vascular permeability factor. Although a definitive role of cytolysin in bacterial infection is controversial (Oliver *et al.*, 1986; Wright & Morris, 1991), *V. vulnificus* cytolysin is still one of the prime candidates in the pathogenesis of disease.

Even sub-microgram amounts of the *V. vulnificus* cytolysin are fatal to mice (Gray and Kreger, 1985). When purified cytolysin was injected into mice, increased vascular permeability and neutrophil sequestration was observed primarily in the lung and resulted in death (Park *et al.*, 1996). The neutrophil is one of the most important components of the host defense system. Patients with leukopenia showed higher mortality rates (Klontz *et al.*, 1988), and the total number of leukocytes has been used as an index of the prognosis of this infectious disease (Park *et al.*, 1991). These findings suggest that neutrophils might be a major target of *V. vulnificus* cytolysin *in vivo*.

We have already reported that the lysis of erythrocytes caused by *V. vulnificus* cytolysin is colloid-osmotic in nature and that cytolysins, after binding to the erythrocyte membrane, oligomerize to form small pores in the membrane resulting in cell lysis (Kim *et al.*, 1993). However, the cytotoxic mechanism against nucleated mammalian cells has not been well characterized. In this

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study, the cytotoxic mechanism of *V. vulnificus* cytolysin was studied in blood neutrophils which might be a nucleated, target cell of *V. vulnificus* cytolysin *in vivo*.

Materials and Methods

Materials Bovine serum albumin, DEAE-cellulose, Freund's complete and incomplete adjuvant, luciferin-luciferase, and Percoll from Sigma Chemical Co. (St. Louis, USA), 2-Deoxy-D-[1,2-^{3}H]glucose from Amersham (Buckinghamshire, England), phenyl-Sepharose CL-4B from Pharmacia LKB Biotechnology (Uppsala, Sweden), Protein A column from Bio-Rad Laboratories (Hercules, USA), heart infusion broth from Gibco Laboratories (Gaithersburg, USA), and cytotoxicity detection kit of lactate dehydrogenase from Boehringer Mannheim (Mannheim, Germany) were used in the experiments. All other reagents were of the highest purity available.

Bacterial strain and culture A virulent strain of *V. vulnificus* E4125 was kindly supplied by Dr. M. H. Kothary (Department of Microbiology, Virulence Assessment Branch, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, USA). The strain was cultured in heart infusion diffusate broth as described by Kreger *et al.* (1988).

Preparation of *V. vulnificus* cytolysin *V. vulnificus* cytolysin was purified to homogeneity from the culture supernatant by a modification of the method developed by Kim *et al.* (1992). The culture supernatant fluids (1000 ml) were obtained by centrifugation (15,000 × *g*, 30 min) after culturing for 4 h at 37°C. Ammonium sulfate was dissolved in the pooled culture supernatant to a final concentration of 55% and the preparation was stored overnight at 4°C. After centrifugation at 10,000 × *g* for 30 min, the precipitate was dissolved and dialyzed in 10 mM glycine-NaOH buffer (pH 9.8) containing 0.05% Nonidet P-40 and 30% glycerol (Solution A). The dialyzed cytolysin was applied to a DEAE-cellulose column (2.5 × 30 cm) equilibrated with Solution A and was eluted with a linear gradient of 0 to 0.3 M KCl in Solution A. Cytolysin was eluted at a concentration of 0.15 M KCl. Pooled cytolysin fractions were directly applied to a phenyl-Sepharose CL-4B column (1.5 × 10 cm) equilibrated with Solution A and unbound proteins were washed out with Solution A. Cytolysin bound on the phenyl-Sepharose CL-4B column was eluted with a linear gradient of 0–30% ethylene glycol in Solution A. Cytolysin was eluted at a concentration of 10% ethylene glycol and was homogenous on an SDS-polyacrylamide gel. The purified *V. vulnificus* cytolysin had a specific activity of 80,000 hemolytic units (HU) per mg of protein with 30% recovery. The hemolytic activity of cytolysin was determined by the methods of Bernheimer and Schwartz (1963).

Preparation of neutrophils Neutrophils were purified from heparinized venous blood of adult Sprague-Dawley rats using the Percoll gradient (55/70%) as described by Wright (1988). After the hypotonic lysis of erythrocytes, purified cells were suspended in Dulbecco's phosphate buffered saline containing 5 mM glucose (DPBS medium) and kept on ice. Cell viability determined by the trypan blue dye exclusion method was over 98%.

Measurement of 2-deoxy-D-[^{3}H]glucose and K^+ efflux The preloading of neutrophils with 2-deoxy-D-[^{3}H]glucose was performed by incubating cells in DPBS medium containing 2-deoxy-D-[^{3}H]glucose (0.25 μCi) in a culture plate under a humidified atmosphere of 5% CO_2 at 37°C for 4 h. After incubation, the supernatant was removed and the cells were washed four times with phosphate buffered saline containing 1 mg/ml bovine serum albumin. The cells were treated with cytolysin in DPBS medium containing 1 mg/ml bovine serum albumin for 5 min at 37°C and centrifuged at 3500 × *g* for 5 min. Radioactivity of the supernatant was measured by a scintillation counter (Kim *et al.*, 1998).

For the measurement of intracellular K^+ release, cells were treated with cytolysin and the medium was collected. The K^+ in the medium was determined by flame spectrophotometry (KLiNa Flame, Beckman) after concentration with a Speed Vac concentrator (Kim *et al.*, 1993).

Current recording Gigaseals were formed with Sylgard-coated pipette (borosilicate, Kimble) with 4 to 5 megaohms resistance and single-channel currents were recorded by use of the inside-out configuration of the patch clamp method described by Hamill *et al.* (1981). Channel currents were recorded with an Axo-patch 1D patch clamp amplifier (Axon) and stored on video tapes via digital recorder (PCM-501ES, Sony) for later computer analysis. A pClamp software package (Axon) was used for data acquisition and analysis. All experiments were carried out at 22 ± 2°C. The standard bath and pipette solutions contained 140 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES (pH 7.4).

Assay of ATP and lactate dehydrogenase After the lysis of cells with 1% Triton X-100 in 20 mM HEPES, cellular ATP was determined by the chemiluminescence method using luciferin-luciferase (Lundin *et al.*, 1979). Twenty microliters of the supernatant, after brief centrifugation, was added to 80 μl of 100 mM glycine buffer (pH 12.6) and 100 μl of luciferin-luciferase solution, and the chemiluminescence was recorded by a chemiluminometer. A standard ATP was prepared just before use.

Lactate dehydrogenase was assayed with a cytotoxicity detection kit according to the manufacturer's instruction (Boehringer Mannheim GmbH, Germany).

SDS-PAGE and immunoblot The oligomer of *V. vulnificus* cytolysin on the neutrophil plasma membrane was detected by a modification of the method developed by Walev *et al.* (1993). Neutrophil suspension (10^7 cells/ml) in DPBS medium was treated with 1.0 μg of cytolysin at 37°C for 30 min. After washing with phosphate buffered saline, the cells were lysed for 30 min on ice in lysis buffer (20 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA). Cellular debris was removed by centrifugation at 100,000 × *g* for 60 min. An aliquot of supernatant was mixed with the same volume of 0.5 M Tris buffer (pH 6.8) containing 4% SDS, 20% glycerol, and 0.05% bromophenol blue at room temperature, and applied to a 7.5% slab gel. SDS-PAGE was performed according to Laemmli (1970). Proteins were transferred to nitrocellulose membrane and immunoblotted with the primary rabbit antibody against *V. vulnificus* cytolysin and alkaline phosphatase-conjugated goat anti-rabbit IgG antibody.

Polyclonal antibody for *V. vulnificus* cytolysin was prepared from New Zealand White rabbit. The rabbit received a primary subcutaneous immunization on the back with 100 μg of purified cytolysin mixed with equal volume of Freund's complete adjuvant. After two subcutaneous booster injections at 4-week intervals, antibodies were purified from sera using a Protein A column.

Results

To determine the binding activity of *V. vulnificus* cytolysin to neutrophils, cells were incubated with cytolysin at 4°C. After brief centrifugation at the indicated time, the hemolytic activity of unbound cytolysin in the supernatant was determined (Fig. 1). The binding of cytolysin to neutrophils occurred rapidly at 4°C, with approximately 70% of cytolysin bound within 1 min. Similar results were obtained at 37°C (data not shown).

Cell viability of neutrophils after *V. vulnificus* cytolysin treatment was determined by the trypan blue exclusion method (Fig. 2). About 50% of neutrophils were killed by treatment with 0.25 HU cytolysin for 30 min at 37°C. As the depletion of cellular ATP is the hallmark of the metabolic deterioration, the cellular ATP level was measured after the cytolysin treatment (Fig. 3). Incubation of neutrophils with 1.0 HU cytolysin caused about 70% of cellular ATP depletion in 1 min. No leakage of cellular ATP was observed in the medium (data not shown). The kinetics of ATP depletion wese closely correlated with the efflux of intracellular K^+. About 90% of potassium was released into the medium in 1 min under the same conditions (Fig. 3).

Neutrophils preloaded with 2-deoxy-D-[^{3}H]glucose were treated with various concentrations of *V. vulnificus* cytolysin. As shown in Fig. 4, nearly all of the preloaded 2-deoxy-D-[^{3}H]glucose was released from the cell by 1.0

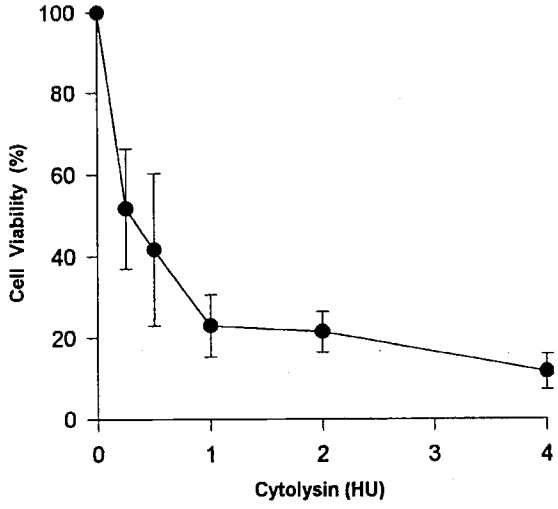

Fig. 2. Effect of *V. vulnificus* cytolysin on the viability of neutrophils. Neutrophils (10^6 cells) were treated with various concentrations of cytolysin in a total volume of 0.3 ml DPBS containing 5 mM glucose and 1 mg/ml bovine serum albumin at 37°C for 30 min. Cell viability was determined by the ability to exclude trypan blue. Each value denotes the mean ± SD obtained from four separate experiments.

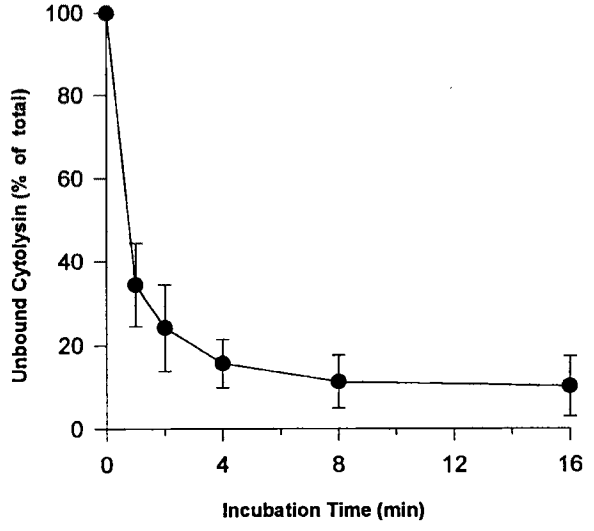

Fig. 1. Binding of *V. vulnificus* cytolysin to neutrophils. Neutrophils (10^6 cells) were incubated with 0.5 HU of the cytolysin in a total volume of 0.3 ml DPBS containing 5 mM glucose and 1 mg/ml bovine serum albumin at 4°C. After brief centrifugation for the indicated time, the residual hemolytic activity in the supernatant was determined as described in Materials and Methods. The plot depicts the mean ± SD obtained from four separate experiments.

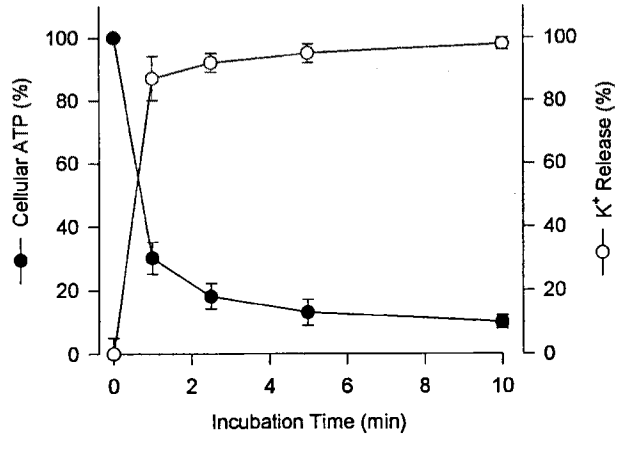

Fig. 3. The time course of cellular ATP depletion and K^+ release by *V. vulnificus* cytolysin. Neutrophils (5×10^6 cells) were incubated with 1 HU of cytolysin in a total volume of 0.3 ml DPBS containing 5 mM glucose and 1 mg/ml bovine serum albumin at 37°C. The cellular ATP content (●) and potassium release (○) at the given times were determined as described in Materials and Methods. Each value denotes the mean ± SD obtained from three separate experiments.

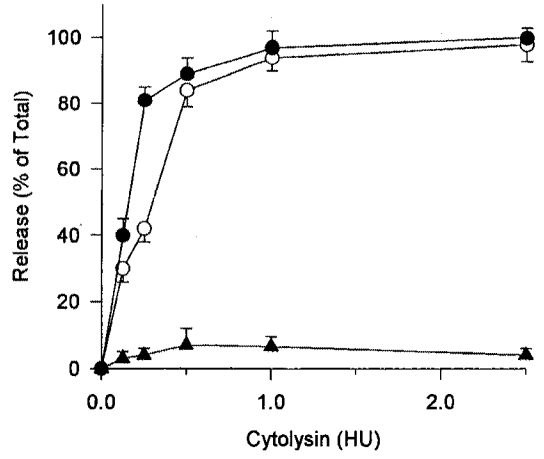

Fig. 4. Effect of *V. vulnificus* cytolysin on the release of 2-deoxy-D-[^{3}H]glucose, K^+, and lactate dehydrogenase from neutrophils. Neutrophils (10^7 cells) preloaded with 2-deoxy-D-[^{3}H]glucose were incubated with various concentrations of cytolysin in a total volume of 1 ml DPBS containing 5 mM glucose and 1 mg/ml bovine serum albumin at 37°C for 5 min. After brief centrifugation, 2-deoxy-D-[^{3}H]glucose (●), K^+ (○), and lactate dehydrogenase (▲) were determined in the supernatant as described in Materials and Methods. The plot depicts the mean ± SD obtained from three separate experiments.

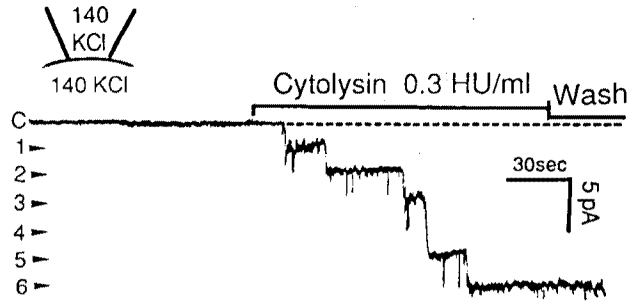

Fig. 5. Effect of intracellularly-applied *V. vulnificus* cytolysin on single channel currents in inside-out patches of neutrophils. The bath and pipette solutions contained 140 mM KCl symmetrically, and the membrane potential was held at −40 mV. Cytolysin was added to the bath solution after the excision of patches. The filter for the tracing reproduction was set at 300 Hz. The number beside the current trace indicates the number of formed pores with C being the closed state.

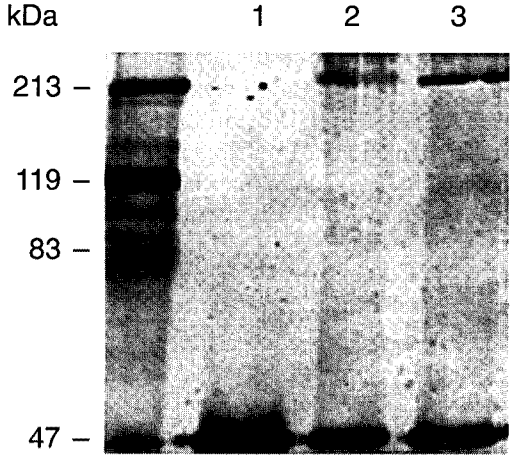

Fig. 6. Oligomerization of *V. vulnificus* cytolysin by neutrophil membranes. Cytolysin (0.1 μg) was incubated at 37°C for 30 min with neutrophils (10^7 cells) or 2 μg cholesterol. Cell lysate was separated by nonreducing SDS-PAGE and immunoblotted with polyclonal rabbit antiserum for cytolysin as described in Materials and Methods. Lane 1, cytolysin alone; lane 2, neutrophil and cytolysin; lane 3, cholesterol and cytolysin.

HU cytolysin within 5 min. K^+ release showed a similar pattern. However, there was no release of lactate dehydrogenase from neutrophils. The formation of transmembrane pores by *V. vulnificus* cytolysin was further investigated with single-channel current recording (Fig. 5). Using the inside-out configuration of the patch clamp method, rapid flow of monovalent ions was observed through the cytolysin-treated neutrophil membrane. These results indicate that *V. vulnificus* cytolysin can induce the formation of small transmembrane pores, which are permeable to monovalent ions or 2-deoxy-D-[^{3}H]glucose, but not to large lactate dehydrogenase.

Oligomerization of the cytolysin monomer was analyzed with nonreducing SDS-PAGE and immunoblotting of neutrophil membranes after treatment with cytolysin (Fig. 6). The membrane-bound cytolysin was detected as a band of M_r 210 kDa, corresponding to a tetramer of native *V. vulnificus* cytolysin of M_r 51 kDa. The molecular weight of oligomer was the same as that of the cytolysin oligomer induced by cholesterol *in vitro*, as reported previously (Kim *et al.*, 1993). These results indicate that the formation of transmembrane pores on the neutrophil membrane was accomplished by the oligomerization of cytolysin monomers.

Discussion

The present study indicates that *V. vulnificus* cytolysin rapidly binds to the neutrophil membrane and oligomerizes to form small transmembrane pores, which induce the efflux of potassium and the depletion of cellular ATP leading to cell death.

Bhakdi & Tranum-Jensen (1988) proposed that generation of transmembrane pores is the mechanism underlying the action of pore-forming exotoxins on target cells. Thiol-activated toxins such as streptolysin O, one of the best characterized examples of pore-forming cytolysins, insert into the target cell membranes to form transmembrane pores (Bhakdi *et al.*, 1985). The cytolysis

by these toxins requires three general steps (Bhakdi & Tranum-Jensen, 1988); (a) binding of toxin to the target cell membrane, (b) penetration and disruption of the membrane (pore formation), and (c) alteration of membrane permeability leading to cytolysis.

V. vulnificus cytolysin bound to neutrophils in a temperature-independent manner and resulted in the cytotoxicity. The pore formation by *V. vulnificus* cytolysin was confirmed by evidence including as the rapid efflux of potassium or 2-deoxy-D-[^{3}H]glucose and the rapid flow of monovalent ions in the patch clamp. The oligomerization of cytolysin monomers on the neutrophil membrane was demonstrated by immunoblot, which exhibited a 210 kDa band, corresponding to a tetramer of the native toxin of M_r 51,000. The pores of neutrophil membranes formed by *V. vulnificus* cytolysin are permeable to only small molecules such as monovalent ions or 2-deoxy-D-[^{3}H]glucose, but not to large molecules such as ATP or lactate dehydrogenase. These findings indicate that *V. vulnificus* cytolysin also induces the formation of small pores on nucleated mammalian cells such as neutrophils, as on erythrocyte membranes (Kim *et al.*, 1993). However, the nucleated cells were resistant to osmotic stress, and the cytolysin did not induce the osmotic lysis of neutrophils, in contrast to the erythrocytes (Kim *et al.*, 1993).

V. vulnificus cytolysin decreased cellular ATP levels similar to other pore-forming cytolysins such as *E. coli* hemolysin (Bhakdi *et al.*, 1986), staphylococcal α-toxin (Harshman *et al.*, 1989), and *V. cholerae* E1 Tor cytolysin (Zitzer *et al.*, 1995). As there was no leakage of cellular ATP from the neutrophils by *V. vulnificus* cytolysin, the reduction of cellular ATP is considered to be due to either an increase in ATP degradation or a decrease in ATP synthesis. The depletion of cellular ATP by *V. vulnificus* cytolysin was closely correlated with the efflux of potassium. It is expected that cellular ATP depletion is derived from the increased consumption of ATP to correct intracellular ionic disturbances. However, Walev *et al.* (1993) demonstrated that the depletion of cellular ATP in keratinocytes by staphylococci α-toxin was accompanied by a decrease in oxygen consumption. These data were interpreted to reflect the breakdown of mitochondrial respiration.

E. coli hemolysin increases the permeability of pulmonary artery endothelial cell monolayers (Suttorp *et al.*, 1990), and bacterial exotoxins stimulate the adhesion of polymorphonuclear leukocytes to endothelial cells in severe local or systemic bacterial infections (Krull *et al.*, 1996). Recently, we have reported that *V. vulnificus* cytolysin caused lethality primarily by increasing vascular permeability and neutrophil sequestration in the lungs of mice (Park *et al.*, 1996). These findings suggest that neutrophils might be the major target cells of *V. vulnificus* cytolysin *in vivo*. Neutrophils are considered as one of the most important components of the immune system for killing invading microorganisms, and the antimicrobial functions of neutrophils are interfered by a variety of bacterial components or products such as the capsular polysaccharide of *V. vulnificus* (Kreger *et al.*, 1981; Shinoda *et al.*, 1987). The resistance of *V. vulnificus* against host defense is especially important when the host is immunocompromised or has underlying disease such as liver cirrhosis. Our results suggest that the potent leukocidal activity of *V. vulnificus* cytolysin might be important in the pathogenesis of *V. vulnificus* infection.

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