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# AN EVIDENCE FOR THE INVOLVEMENT OF CYTOLYSIN IN *VIBRIO VULNIFICUS* DISEASE

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***ABSTRACT:*** *Cytolysin produced by Vibrio vulnificus ATCC 27562 was partially purified by sequential ammonium sulfate precipitation, gel filtration with Sephadex G-200, and ion exchange chromatography with DEAE-Sephadex. The partially purified cytolysin was inactivated by cholesterol. More than one molecule of the cytolysin was required to lyse a single erythrocyte. The antiserum against cytolysin enhanced the survival ratio of mice infected with low dose of V. vulnificus.*
***Keywords:*** *Cytolysin, Vibrio vulnificus*

## INTRODUCTION

*Vibrio vulnificus* is a gram-negative, halophilic bacterium that is capable of causing rapidly progressing, life-threatening wound infections and septicemia in humans (Blake *et al.*, 1980). Putative virulence factors produced by *V. vulnificus* include extracellular cytolysin (Kreger and Lockwood, 1981), proteases and phospholipases (Testa *et al.*, 1984), siderophores (Simpson and Oliver, 1983), and antiphagocytic surface antigens (Yoshida *et al.*, 1985). Gray and Kreger (1985) showed that purified cytolysin is cytotoxic to chinese hamster ovary cells, hemolytic for mammalian erythrocytes, and lethal for mice. Most clinical strains of *V. vulnificus* have been reported to produce cytolysin when grown under appropriate *in vitro* conditions, but whether the cytolysin is actually produced *in vivo* and has an important role in the pathogenesis of *V. vulnificus* disease is not yet known.

In the present study, the production of cytolysin *in vivo* was indirectly examined by determining whether the antiserum raised against cytolysin can contribute to the survival of mice infected with *V. vulnificus*. In addition, some properties of the cytolysin were examined.

## MATERIALS AND METHODS

### Organism

*V. vulnificus* ATCC 27562 was purchased from American Type Culture Collection

(Maryland, USA). Stock cultures were prepared by growing the organism in heart infusion broth (Difco) and freezed with sterile 15% glycerol at -70°C. The stock culture was then inoculated into heart infusion agar slant. After incubation for 24 hours at 37°C, the agar slant was washed off with 5 m*l* of sterile 0.85% NaCl, and the suspension was adjusted to an optical density at 650 nm of ca. 10. One liter flask containing 200 m*l* of heart infusion broth was inoculated with 1 m*l* of the suspension and incubated at 37°C with vigorous shaking for 6 hours.

**Purification of cytolysin**

All the procedures for the purification of cytolysin were carried out at 4°C.

Culture supernatant fluids were obtained by centrifugation (15,000 × g, 15 min.) after incubation of culture. Solid ammonium sulfate was dissolved in the pooled culture supernatant fluids (1,200 m*l*) to a concentration of 50% saturation. After 16 hours, the precipitate was recovered by centrifugation (15,000 × g, 15 min.) and dissolved in 100 m*l* of 10 mM imidazole buffer, pH 6.6. The solution was centrifuged (15,000 × g, 15 min.) to remove a small amount of insoluble residues and saturated ammonium sulfate solution was added to the supernatant to a concentration of 40% saturation. After 16 hours, the precipitate was recovered by centrifugation (15,000 × g, 15 min.) and dissolved in 10 m*l* of the same buffer. Insoluble residues were removed from the solution by centrifugation.

A volume of 10 m*l* was then applied to a column (2.6 by 95 cm) of Sephadex G-200 (Pharmacia) equilibrated with the same buffer and eluted at a flow rate of 12 m*l* per hour. Fractions (4 m*l*) were assayed for absorbance at 280 nm and for cytolytic activity. The cytolysin peak fractions were pooled.

NaCl was added to the cytolysin pool (ca. 32 m*l*) to a final concentration of 200 mM and this solution was applied to a column (1.6 by 30 cm) of DEAE-Sephadex A-50 (Pharmacia) equilibrated with 10 mM imidazole buffer, pH 6.6 supplemented with 200 mM NaCl. The column was washed (30 m*l*/h) with ca. 100 m*l* of 10 mM imidazole buffer, pH 6.6 supplemented with 200 mM NaCl. The cytolysin was eluted (20 m*l*/h) with 10 mM imidazole buffer, pH 6.6 containing 400 mM NaCl. The fractions (3 m*l*) were assayed for absorbance at 280 nm and for cytolytic activity. Pooled cytolysin preparation (ca. 18 m*l*) was used in the following experiments.

**Assays**

For cytolysin assay, the hemolytic activity of samples was determined with mouse erythrocytes. Cytolysin dilutions were prepared with phosphate-buffered saline (0.067 M $Na_2HPO_4$, 0.077 M NaCl, pH 7.0) containing 1 mg of bovine albumin (Sigma) per m*l* (PBS-BA). Collected mouse erythrocytes were washed twice and diluted with PBS-BA to a concentration of 0.7% (v/v). Serial dilutions, 100 u*l*, of cytolysin sample were pipetted into adjacent wells of 96-well microtiter plates and 50 u*l* of 0.7% (v/v) mouse erythrocyte suspension was added. The plates were incubated at 37°C for 30 minutes and then examined visually for hemolysis. The hemolytic unit of a sample was defined as the reciprocal of the highest dilution causing complete hemolysis.

Protein was estimated by the method of Bradford (1976) with bovine albumin (Sigma) as the standard.

### Characterization of cytolysin

The effect of cholesterol on the cytolysin was examined by incubating the partially purified cytolysin samples (100 HU in 1 m*l* of PBS) with various amounts of cholesterol (Sigma) at 37°C for 15 minutes and assaying the mixtures for residual cytolytic activity.

The effect of erythrocyte concentration on erythrocyte lysis was studied. Washed mouse erythrocyte suspensions (1 m*l*) of various concentrations were incubated with the partially purified cytolysin (2.5 HU in 1 m*l* of PBS-BA) at 37°C for 30 minutes, the mixtures were centrifuged to sediment the unlysed erythrocytes, and the absorbance of the supernatant was determined at 545 nm.

### Preparation of antiserum

New Zealand white rabbits, weighing ca. 2.5 kg each at the start of vaccination schedule, were injected subcutaneously with 0.7 m*l* of cytolysin preparation (100 HU) suspended in complete Freund's adjuvant. After 4 weeks, the rabbits were reinjected subcutaneously with 0.5 m*l* of cytolysin preparation (50 HU) suspended in incomplete Freund's adjuvant. The rabbits were exanguished 3 weeks after the second injection and the pooled sera were tested for cytolysin-neutralizing activity and stored at-20°C.

### Toxicity tests

Mice (ICR strain) of 20 to 25 g were used in the toxicity tests. The bacterial suspension used to challenge the mice was prepared by growing the bacteria in heart infusion broth, collecting the bacteria by centrifugation, and suspending the bacteria in 0.85% NaCl. 0.1 m*l* of two-fold dilutions of the bacteria suspension was injected subcutaneously in groups of 10 mice per group. The $LD_{50}$ value was estimated by the method of Reed and Muench (1938) after observing the animals for 3 days post-injection.

Protective effects of rabbit anti-cytolysin serum were studied in mice. Groups of mice (30 mice pre group) were challanged with 1, 5, 10 or 100 $LD_{50}$ of *V. vulnificus*, s.c., and 0.1 m*l* of antiserum was injected i.v. right after the challenge and every 12 hrs thereafter. Mice were observed for 3 days after the challenge.

## RESULTS AND DISCUSSION

### Partial purification of cytolysin

Fig. 1 shows the result of gel filtration on Sephadex G-200. The cytolysin was eluted in the void volume. Fig. 2 shows the result of ion exchange chromatography on DEAE-Sephadex. The cytolysin binded to DEAE-Sephadex at 10 mM imidazole buffer, pH 6.6 containing 200 mM NaCl and eluted in a sharp peak when 400 mM NaCl was supplemented. Results of the purification scheme are summarized in Table 1.

### Characteristics of cytolysin

The cytolysin was almost totally inactivated when 100 ug of cholesterol was added

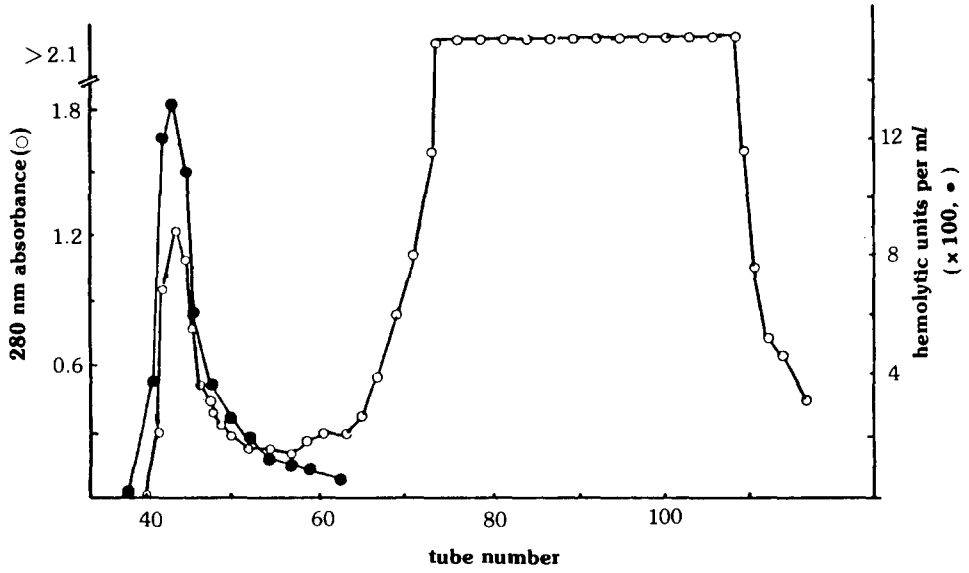


**Fig. 1.** Gel filtration on Sephadex G-200 column (2.6 by 95 cm) equilibrated with 10 mM imidazole buffer, pH 6.6. 10 m*l* of sample was applied and eluted at a flow rate of 12 m*l* per hour. Fraction size was 4 m*l*.

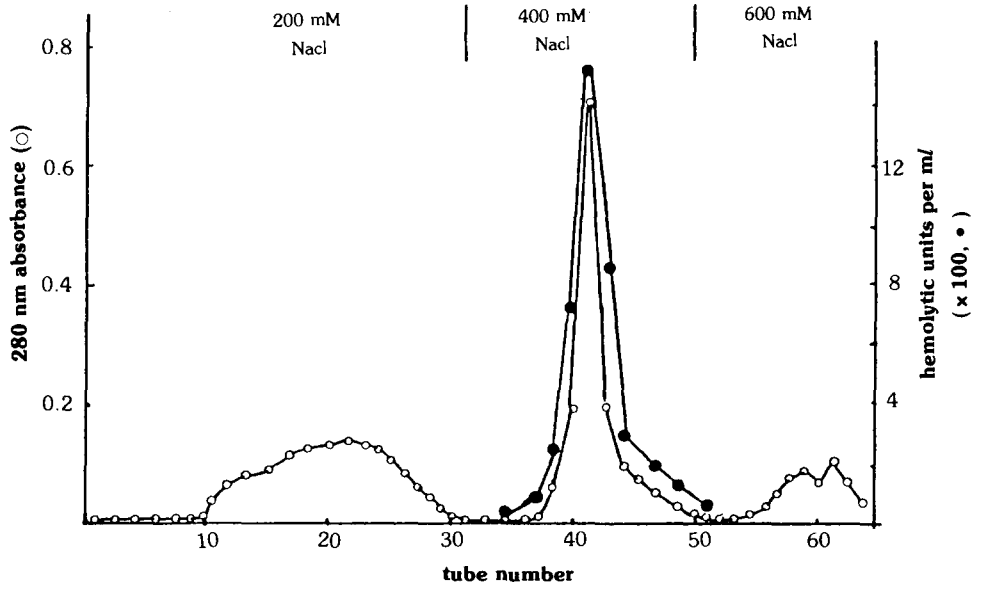


**Fig. 2.** DEAE-Sephadex column chromatography. 32 m*l* of sample was applied to a column (1.6 by 30 cm) and eluted sequentially with 10 mM imidazole buffer, pH 6.6 supplemented with 200 mM NaCl, 10 mM imidazole buffer, pH 6.6 supplemented with 400 mM NaCl, and 10 mM imidazole buffer, pH 6.6 supplemented with 600 mM NaCl. Fraction size was 3 m*l*.

**Table 1.** Partial purification of cytolysin produced by *Vibrio vulnificus* ATCC 27562.

| Purification stage | Volume (ml) | Protein (mg/ml) | Total protein (mg) | Activity (HU/ml) | Total activity (HU) | Recovery (%) | Specific activity (HU/mg protein) |
|---|---|---|---|---|---|---|---|
| Culture supernatant | 1,200 | 0.29 | 348 | 42 | 50,400 | 100 | 145 |
| Ammonium sulfate precipitation | 10 | 13 | 130 | 4,200 | 42,000 | 83.3 | 323 |
| Sephadex G-200 gel filtration | 32 | 0.36 | 11.5 | 780 | 24,900 | 50 | 2,170 |
| DEAE-Sephadex chromatography | 18 | 0.13 | 2.34 | 730 | 13,140 | 26 | 5,615 |

(Table 2). This result suggests that the cytolysin of *V. vulnificus* ATCC 27562 may interact with cholesterol-containing site of erythrocyte membrane similar to the oxygen-labile, thiol-activated bacterial exotoxins including tetanolysin, cereolysin, listeriolysin, perfringolysin, and streptolysin-O.

The effect of erythrocyte concentration on hemolysis is shown in Fig. 3. The amount of hemolysis reached a peak at the erythrocyte concentration of 0.75% and then decreased. These observations suggest that more than one molecule of the cytolysin is required to lyse a single erythrocyte (Inoue *et al.*, 1979).

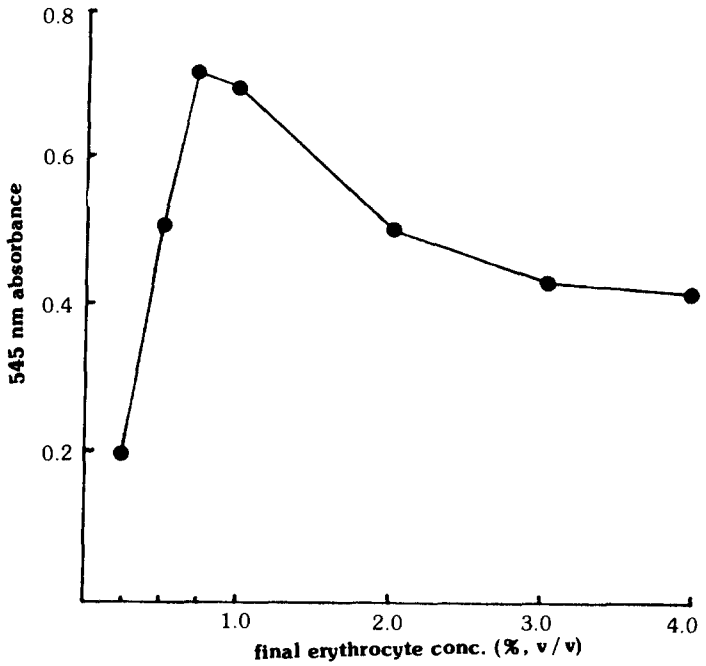


**Fig. 3.** Effect of erythrocyte concentration on hemolysis by *Vibrio vulnificus* cytolysin. Samples (1 ml) of mouse erythrocyte suspensions of various concentrations were incubated with partially purified cytolysin (2.5 HU in 1 ml of PBS-BA) at 37°C for 30 minutes. The mixtures were centrifuged to sediment the unlysed erythrocytes, and absorbance of supernatant was determined at 545 nm.

**Table 2.** The effect of cholesterol on cytolysin activity.

| amount of cholesterol added ($\mu$g) | residual activity (HU/ml) |
|---|---|
| 100 | 1 |
| 50 | 20 |
| 25 | 30 |
| 1 | 60 |

Cytolysin samples (100 HU in 1 ml of PBS) were incubated with 1, 25, 50, and 100 $\mu$g of cholesterol at 37°C for 15 minutes and the mixtures were assayed for residual cytolysin activity.

**Table 3.** Passive immunization of mice against *Vibrio vulnificus* disease by vaccination with antiserum against cytolysin.

| Challenge dose | % survival after injection of | |
|---|---|---|
| | normal rabbit serum | antiserum |
| 100 $LD_{50}$ | 0 | 0 |
| 10 $LD_{50}$ | 3 | 7 |
| 5 $LD_{50}$ | 10 | 17 |
| 1 $LD_{50}$ | 47 | 77 |

Membrane-filter sterilized antiserum (0.1 ml) was injected intravenously into groups of mice (30 per group) zero time and every 12 hours after subcutaneous challenge with various amounts of *V. vulnificus*. The mice were observed for 3 days postchallenge.

### Protective activity of anti-cytolysin serum

*V. vulnificus* ATCC 27562 injected subcutaneously into mouse had a 50% lethal dose value of $3.5 \times 10^7$ colony forming units. The antiserum against cytolysin had an activity of neutralizing 128 hemolytic units per ml. The results of the passive immunization tests after challenge with 1, 5, 10, and 100 $LD_{50}$ of *V. vulnificus* are shown in Table 3. When more than 5 $LD_{50}$ of *V. vulnificus* was challenged, the antiserum against cytolysin had little effect to the survival of the mice. These results may be due to the fact that the cytolysin is not the only virulence factor *V. vulnificus* produces or that the antibody level of antiserum injected was not sufficient to neutralize all the cytolysin that is produced in the mouse. However, when low dose of *V. vulnificus* was challenged, the antiserum contributed, in a some extent, to the survival of the mice. Although these results are not direct evidences, they suggest that the cytolysin is not only produced in the mouse but also involved in the pathogenesis of *V. vulnificus* disease.

## ACKNOWLEDGEMENT

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