

Phospholipase A as a potent virulence factor of *Vibrio vulnificus*

BON-SUN KOO^{1*}, JI-HYUN LEE^{1*}, SOON-CHEOL KIM¹, HA-YONG YOON¹, KYUNG-AH KIM¹,
KANG-BEOM KWON², HYUNG-RHO KIM¹, JIN-WOO PARK¹ and BYUNG-HYUN PARK¹

¹Department of Biochemistry, Medical School and Institute for Medical Sciences, Chonbuk National University, Jeonju, Jeonbuk 561-756; ²Department of Physiology, School of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk 570-749, Korea

Received August 9, 2007; Accepted September 24, 2007

Abstract. *Vibrio vulnificus* infection has attracted special interest because of its high mortality rate. However, the identification of its major pathogenic determinant still remains obscure. In this study, a cytolysin-negative mutant strain of *V. vulnificus* CVD707 was used to determine the role of phospholipase A (PLA) in the pathogenesis of this bacterial infection. The mutant strain caused the lysis of erythrocytes *in vitro* and elevated plasma hemoglobin during the infection in mice. Both the hemolytic and PLA activities were dependent on calcium. Inhibition of hemolysis by PLA inhibitors including tetracyclin and the PLA substrate phosphatidylcholine also supports the possibility of membranous PLA as a major hemolytic factor in the cytolysin-deficient mutant. To identify the role of PLA in the pathogenesis of *V. vulnificus* infection, the effects of tetracycline on bacteria-induced macrophage cytotoxicity and lethality were compared with those of penicillin, an antibiotic with no inhibitory effect on PLA. Both the macrophage cytotoxicity and the lethality of *V. vulnificus* CVD707 to mice were significantly attenuated by tetracycline, but not by penicillin. However, bacterial counts in culture medium and mouse blood revealed that penicillin was more effective than tetracycline in killing bacteria under our experimental conditions. These results indicate that PLA activity is important in *V. vulnificus*-induced cytotoxicity and lethality, suggesting a crucial role for PLA in the pathogenesis of *V. vulnificus* infection.

Introduction

Vibrio vulnificus is an estuarine Gram-negative bacterium that causes severe wound infection and fulminant septicemia (1). The infection caused by this bacterium has attracted special attention because of its high patient mortality (2,3). Various factors have been implicated as contributors to the disease caused by *V. vulnificus*. One of these factors is the presence of a cytolysin that causes cell lysis and vascular permeability enhancement (4). This species is also believed to be dependent on the availability of iron in human serum for their survival (5). Other disease factors may include protease (6), phospholipase (7), or the presence of a polysaccharide capsule (8). However, the identity of the major pathogenic determinant still remains obscure.

It is well known that *V. vulnificus* infection is greatly dependent on the blood iron level of the host. Wright *et al* (5) showed that iron overload in mice greatly reduced the 50% lethal dose of *V. vulnificus*. Suppressed bacterial growth in iron-deficient medium can be restored by the addition of heme protein or iron-saturated transferrin (9). Epidemiological studies also show that fatal sepsis of *V. vulnificus* is strongly associated with underlying liver diseases such as liver cirrhosis or hemochromatosis, all of which often cause elevated serum iron levels (2,10-12).

V. vulnificus is able to utilize various heme-containing compounds, including hemoglobin, as *in vitro* and *in vivo* iron sources (13,14). These findings suggest that hemolytic factors produced by the bacterium may play an important role in the pathogenesis of septicemia. *V. vulnificus* cytolysin, originally identified as hemolysin (15), causes the lysis of erythrocytes through the formation of small pores on the cell membrane (16). The capability to produce cytolysin is associated with the virulence of the bacterial strain (17,18). Cytolysin itself is extremely toxic, and even a submicrogram amount of purified cytolysin is fatal to mice when injected intravenously (3). The toxicity of *V. vulnificus* cytolysin is not confined to erythrocytes. It also acts on endothelial and other eukaryotic cells by inducing apoptotic cell death (19). However, the cytolysin-negative mutant strain of *V. vulnificus* still retained its lethal activity on mice (20), suggesting that the role of cytolysin appears to be less important than other factors in the pathogenesis of *V. vulnificus* infection.

Correspondence to: Dr Byung-Hyun Park, Department of Biochemistry, Medical School and Institutes for Medical Sciences, Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea
E-mail: bhpark@chonbuk.ac.kr

*Contributed equally

Key words: phospholipase A, hemolysis, tetracycline, calcium, *V. vulnificus*

In this study, using the cytolysin-negative mutant strain of *V. vulnificus*, we observed that significantly high hemolytic activity was still retained *in vitro*, and that plasma hemoglobin was also elevated in infected mice. Seeking the new hemolytic factor in the mutant strain, we found that the major hemolytic and lethal factor was phospholipase A (PLA).

Materials and methods

Bacterial strain and animals. CVD707, a cytolysin-negative mutant strain of *V. vulnificus*, was kindly donated by J.G. Morris Jr (Division of Infectious Disease, University of Maryland, School of Medicine, Baltimore, MD). Bacteria were cultured in brain heart infusion diffusate broth (Gibco Invitrogen, Carlsbad, CA) at 37°C for 4 h as described by Kreger *et al.* (4). Specific pathogen-free female ICR mice were purchased from the Korean Research Institute of Chemical Technology (Daejeon, Korea) and housed in our animal facility for one week before use. All the mice used were 5-6 weeks old. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Chonbuk National University.

Assay of plasma hemoglobin. *V. vulnificus* CVD707 (1×10^7 CFU) diluted in 100 μ l of phosphate-buffered saline (PBS) was injected intraperitoneally into mice. At the indicated time, blood samples were collected by heart puncture, immediately transferred to heparinized microfuge tubes, and briefly centrifuged to obtain plasma. Plasma hemoglobin was measured by its myeloperoxidase activity with the method of Anderson *et al.* (21). Plasma (100 μ l) was added to 1.4 ml of 5 mM potassium phosphate buffer (pH 6.0) containing 0.005% H_2O_2 and 0.234 mg O-dianisidine. A_{460} was measured after a 3-min incubation.

Assay of hemolytic activity. The hemolytic activity of the bacteria or their lysate against mouse erythrocytes was determined by the method of Bernheimer and Schwartz (22). *V. vulnificus* CVD707 (5×10^{10} CFU/ml of 50 mM Tris-HCl, pH 8.0) was lysed by ultrasonification for 2 min on ice. Intact bacteria or their lysate were mixed with mouse erythrocyte suspensions (0.75% in 10 mM $CaCl_2$, 0.9% NaCl, and 50 mM Tris-HCl, pH 8.0), and incubated at 37°C for 60 min. After brief centrifugation, released hemoglobin in the supernatant was determined by reading the absorbance at 545 nm.

Assay of PLA activity. The PLA activity was determined by the modification of a radioassay developed by Lumb *et al.* (23). One μ Ci of ^{14}C -phosphatidylcholine (Amersham, Buckinghamshire, UK) and 1.7 μ mol of unlabelled phosphatidylcholine were dissolved in chloroform and then evaporated. The substrate was re-suspended in 1 ml of 0.1% deoxycholate, 10 mM $CaCl_2$, and 0.1 M Tris-HCl buffer, pH 8.0, by vigorous vortexing. Intact bacteria or their lysate and substrate solution (15 μ l) were added to 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1% deoxycholate and 10 mM $CaCl_2$ (total volume, 200 μ l), and incubated for 3 h at 37°C in a shaking water bath. After addition of 1 ml of fatty acid extraction mixture (toluene:chloroform:methanol, 1.0:0.5:1.2, v:v:v) and 20 μ l of 1 N NaOH, the samples were vigorously

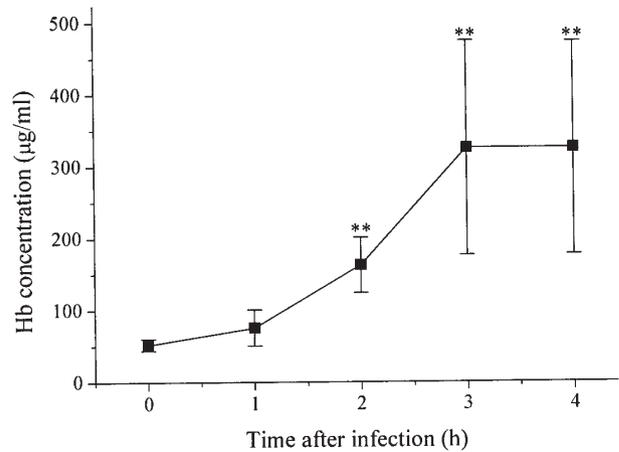


Figure 1. Change of plasma hemoglobin in mice infected with cytolysin-deficient *V. vulnificus*. Mice were infected with an intraperitoneal injection of *V. vulnificus* CVD707 (10^7 CFU). Blood was taken at the indicated times, and the plasma hemoglobin (Hb) level was determined by the myeloperoxidase method. Each point is the mean \pm SE obtained from 4 animals. ** $P < 0.01$ vs. time 0.

vortexed and centrifuged at 1,000 \times g for 10 min to separate the aqueous and organic phases. The radioactivity in aqueous phase (400 μ l) was determined by a scintillation counter.

Preparation of peritoneal macrophage and cytotoxicity assay. Murine macrophages were obtained from ICR mice that had been injected with 2 ml of sterile 3% thioglycolate (24). Four days after injection, peritoneal exudate cells were harvested by peritoneal lavage with 6 ml of ice-cold PBS per mouse. These cells were washed twice and then suspended at 5×10^6 cells/ml in DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin G, and 5 μ g/ml streptomycin (working medium). After a 2-h incubation in 75-cm² culture flasks at 37°C in a humidified 95% air/5% CO_2 atmosphere, nonadherent cells were removed by repeated washing with ice-cold PBS. Adherent cells (peritoneal exudate macrophage) were harvested using a cell scraper and re-suspended at 10^6 cells/ml in working medium. The macrophages obtained (10^6 cells) in this step were incubated with *V. vulnificus* CVD707 in 300 μ l DMEM at 37°C for 3 h, and cell viability was determined by the ability to exclude trypan blue (25).

Statistical analysis. Statistical analysis of the data was performed with the Student's t-test and ANOVA followed by the Duncan's test. Differences of $P < 0.05$ were considered statistically significant.

Results

The bacterial strain used in these experiments was CVD707, a cytolysin-negative mutant strain of *V. vulnificus* (20). To determine whether hemolysis was still induced in mice infected with a cytolysin-deficient strain, plasma hemoglobin levels were measured after bacterial injection. As shown in Fig. 1, plasma hemoglobin levels were significantly increased in a time-dependent manner after bacterial injection. This finding suggests that iron overload through hemolysis still

Table I. Existence of PLA and hemolytic activities of cytolysin-deficient *V. vulnificus*.

Fractions	PLA activity (%)	Hemolytic activity (%)
Intact <i>V. vulnificus</i> CVD707	100	100
<i>V. vulnificus</i> CVD707 lysate	104.5±7.6	100.9±13.4
Heat-treated <i>V. vulnificus</i> CVD707 lysate	3.3±0.6 ^a	ND

The results are expressed as the mean ± SE of 4 separate experiments. ND, not detectable. ^aP<0.01 vs. intact *V. vulnificus* CVD707.

Table II. Agents inhibiting PLA and hemolytic activities of cytolysin-deficient *V. vulnificus*.

Inhibitors	PLA activity (%)	Hemolytic activity (%)
None	100	100
Phosphatidylcholine, 100 µg/ml	6.0±3.9 ^b	10.5±0.7 ^b
Streptomycin, 1 mM	30.1±4.1 ^a	14.3±5.1 ^b
Quinacrine, 1 mM	26.3±3.7 ^a	17.3±5.1 ^b
4-Bromophenacyl bromide, 1 mM	67.2±11.0	ND
Quercetin, 1 mM	90.0±21.8	79.6±14.3
Tetracyclin, 10 µM	40.1±6.7 ^a	21.4±9.2 ^a
Penicillin, 1 mM	96.4±7.1	93.9±2.0

The results are expressed as the mean ± SE of 4 separate experiments. ND, not determined because of the reagent's own hemolytic activity. ^aP<0.05, ^bP<0.01 vs. none.

occurs in the absence of cytolysin, and other hemolytic factors may participate in hemolysis caused by mutant bacteria.

Intact bacteria and lysate of the cytolysin-deficient strain were both equally able to cause the lysis of erythrocytes *in vitro* (Table I). The bacterial lysate was extremely heat-labile, showing complete loss of hemolytic and PLA activities even at 50°C for 10 min.

The effects of various PLA inhibitors on the hemolytic and PLA enzyme activities of *V. vulnificus* CVD707 lysate are presented in Table II. The presence of excess phosphatidylcholine, a substrate of phospholipase, competitively inhibited the hemolytic activity of intact bacteria, suggesting that phospholipase was a possible hemolytic factor. Inhibition of PLA activities with tetracycline (26), quinacrine (27), quercetin (28), 4-bromophenacyl bromide (28), or streptomycin (29) was observed with varying degrees, and these changes in PLA activity were well correlated with changes in hemolytic activity.

Among the inhibitors used, tetracycline was most potent (Table II). Dose-dependent inhibition of phospholipase A

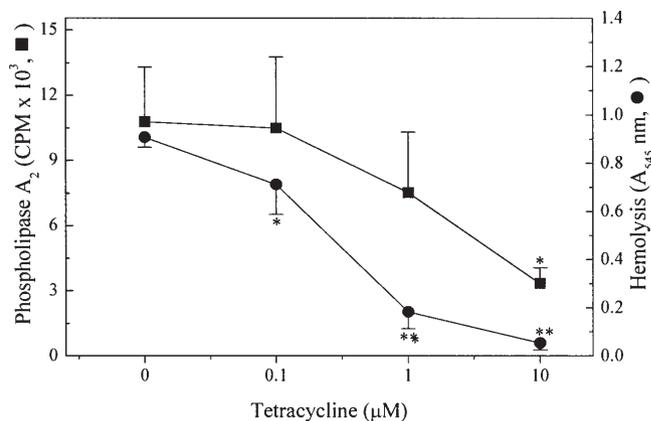


Figure 2. Inhibitory effect of tetracycline on PLA and hemolytic activities of cytolysin-deficient *V. vulnificus*. PLA and hemolytic activities of *V. vulnificus* CVD707 lysate were determined at various concentrations of tetracycline as described in Materials and methods. The results are expressed as the mean ± SE of 3 separate experiments. *P<0.05, **P<0.01 vs. none.

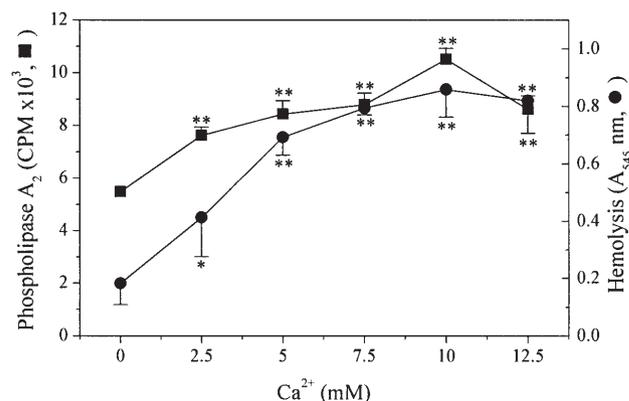


Figure 3. Effect of calcium on PLA and hemolytic activities of cytolysin-deficient *V. vulnificus*. PLA and hemolytic activities of *V. vulnificus* CVD707 lysate were determined at various concentrations of Ca²⁺ as described in Materials and methods. The results are expressed as the mean ± SE of 3 separate experiments. *P<0.05, **P<0.01 vs. none.

activity with tetracycline was accompanied by the inhibition of hemolysis in a concentration-dependent manner (Fig. 2). Most PLA enzymes require calcium for their activation (15). As shown in Fig. 3, both the hemolytic and PLA activities of bacterial lysate were greatly enhanced by calcium in the reaction mixture. These results again strongly support the possibility of PLA as a major hemolytic factor in this cytolysin-deficient *V. vulnificus* mutant strain.

Our next step was to define the role of PLA as a virulence determinant in *V. vulnificus* CVD707 infection. Therefore, we investigated the effects of PLA inhibitors on *V. vulnificus* CVD707-induced cytotoxicity of macrophages and bacterial viability. However, the effective concentrations for most of these PLA inhibitors were much higher than that of tetracycline; high enough to cause severe toxicity in cell and animal studies (Table II). As tetracycline is an antibiotic compound, another antibiotic, penicillin, was chosen for comparison studies. Penicillin exhibited no inhibition of PLA

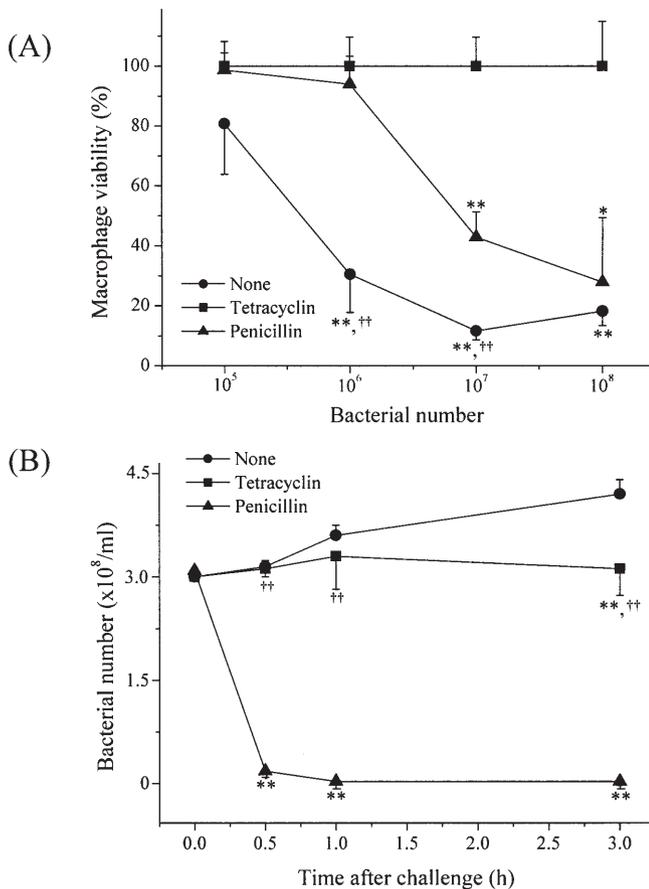


Figure 4. Effects of tetracycline and penicillin on cytolysin-deficient *V. vulnificus*-induced macrophage damage (A) and bacterial viability (B). Murine macrophages (10^6 cells) were incubated with an indicated amount of *V. vulnificus* CVD707 at 37°C for 3 h in the presence or absence of 10 μ M tetracycline or 50 μ M penicillin in 300 μ l DMEM, and cell viability was determined by the ability to exclude trypan blue (A). To check bacterial susceptibility to antibiotics, *V. vulnificus* CVD707 (10^7 CFU) were incubated at 37°C for the indicated time in the presence or absence of 10 μ M tetracycline or 50 μ M penicillin in 300 μ l DMEM, and the number of colonies was counted after culturing at BHI agar at 37°C overnight (B). Each value denotes the mean \pm SE of 3 experiments. * P <0.05, ** P <0.01 vs. none; † P <0.01 vs. the penicillin-treated group.

and hemolytic activities even at a 1-mM concentration. But tetracycline, at a concentration 1/100th that of penicillin, significantly inhibited PLA and hemolytic activities (Table II).

When murine macrophages (10^6 cells) were incubated with *V. vulnificus* CVD707 (10^7 CFU) in culture medium, ~90% of cells were dead within 3 h (Fig. 4A) and the number of bacteria was gradually increased during the culture (Fig. 4B). In the presence of 50 μ M penicillin, this cytotoxicity to macrophages was somewhat attenuated, but still persisted; $42.8 \pm 8.6\%$ of the macrophages were still alive at bacterial concentrations of 10^7 CFU in the culture medium (Fig. 4A). However, most of the bacteria were dead at 30 min after culture with penicillin (Fig. 4B). In contrast, cytotoxicity against macrophages was completely prevented by 10 μ M tetracycline at bacterial counts ranging from 10^5 to 10^8 CFU (Fig. 4A). The number of viable bacteria in the medium remained static until 3 h (Fig. 4B).

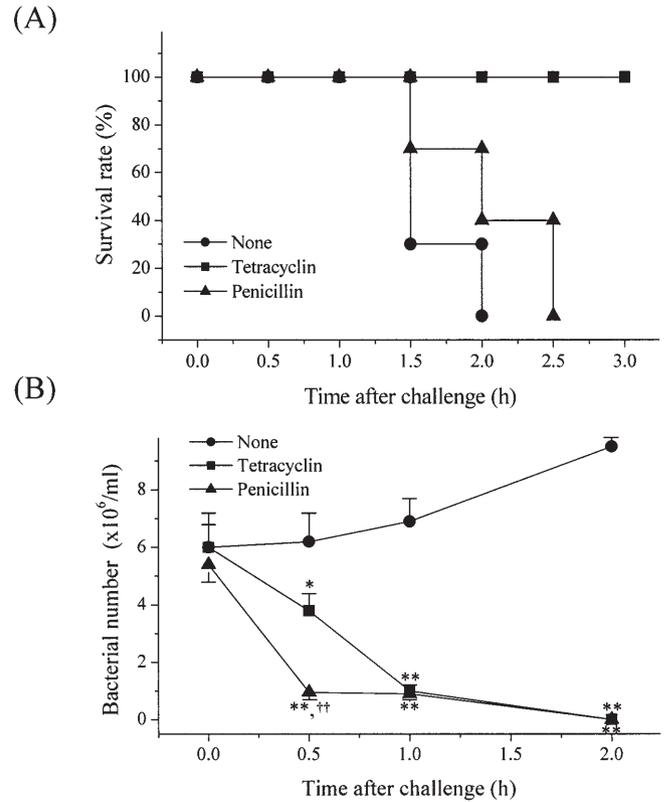


Figure 5. Effect of tetracycline and penicillin on lethality by cytolysin-deficient *V. vulnificus* (A) and blood bacterial number (B) in mice. Mice (10 for each group) were treated with vehicle, tetracycline (5 mg/kg i.v.) or penicillin (25 mg/kg i.v.) just before the intraperitoneal injection of *V. vulnificus* CVD707 (10^8 CFU), and their survival was monitored for 3 h (A). For blood bacterial counting, blood was taken at the indicated time and cultured at BHI agar at 37°C overnight, and the number of colonies was counted (B). Each value denotes the mean \pm SE of 4 mice. * P <0.05, ** P <0.01 vs. none; † P <0.01 vs. the penicillin-treated group.

Similar results were observed for the mortality of mice infected with *V. vulnificus* CVD707. At a dosage of 10^8 CFU, all mice were dead in 2 h (Fig. 5A), and the number of viable bacteria in the blood was greatly increased (Fig. 5B). A prior injection of penicillin killed most of the bacteria in the blood within 30 min (Fig. 5B), but could not prevent death caused by bacterial infection (Fig. 5A). The bactericidal efficiency of tetracycline was weaker than that of penicillin (Fig. 5B), however, all the mice were alive under the same conditions of infection (Fig. 5A). These results suggest that PLA activity is an important determinant in *V. vulnificus* CVD707-induced cytotoxicity and lethality.

Discussion

Iron increases the growth rate of *V. vulnificus* in cultures as well as in mice (13,30), and hemoglobin released through hemolysis is an important source of plasma iron (13,14). The role of iron in the animal host is thought to greatly facilitate the proliferation of *V. vulnificus*, increasing the bacterial number beyond the capability of the mouse defense system to effectively reduce it. The resultant high numbers of bacteria cause death (30). The final mechanism of lethality therefore appears to be independent of iron. Such toxicity could be

caused by the increase in some toxic factors produced by large numbers of bacteria. *V. vulnificus* cytolysin was thought to be a potent virulence determinant in bacterial infection (3) and was originally identified as a hemolysin causing the lysis of cells (31). In this study, we used CVD707, a cytolysin-negative mutant strain of *V. vulnificus*. Wright and Morris (20) used the same strain, and reported that it was still lethal to mice. In spite of the mutation to the cytolysin gene, elevation of plasma hemoglobin was still observed during infection with the mutant strain. Furthermore, the mutant bacteria itself can cause lysis of erythrocytes *in vitro*. These results indicate that a new hemolytic factor is present in the cytolysin-negative mutant strain, and it provides enough cell-free blood iron for bacterial growth.

To aid in the invasion of host tissues, microbial cells possess constitutive and inducible hydrolytic enzymes that destroy or derange constituents of host cell membranes, leading to membrane dysfunction and/or physical disruption. By cleaving phospholipids, phospholipases destabilize the membrane resulting in cell lysis. Evidence implicating phospholipase in host cell penetration, injury, and lysis by microorganisms has been reported in various types of bacteria and fungi (32,33). Consequently, phospholipases have been included among the virulence factors that damage host cells.

The bacterial phospholipases comprise a diverse group of proteins that are considered to be virulence factors for bacterial species that cause serious diseases. Such illnesses range from infections causing massive tissue destruction, such as gas gangrene and lung infections of *Pseudomonas aeruginosa*, to food-borne listeriosis (32-34). Triggering bacterial entry, endosomal lysis, and cytolysis to modulate the local immune response and stimulating cytokine secretion (34) were also suspected to be action mechanisms of phospholipases in diseases. The requirement of calcium as a cofactor for enzymatic activity of outer membranous forms of PLA (OMPLA) has been reported in Gram-negative bacteria. Its activity is calcium-dependent and strictly regulated by reversible dimerization (9,15,35,36). Our enzyme was also calcium-dependent. PLA activity was determined using a radioactive fatty acid attached at the *sn*-2 position of glycerol. The identity of our PLA and its relationship with the already reported OMPLA and the specificity of *V. vulnificus* phospholipase are both topics that need further study. Strong inhibition of hemolysis by PLA inhibitors including tetracycline and phosphatidylcholine was observed in this study. Such data also support the possibility that membranous PLA of *V. vulnificus* is a major hemolytic factor in a cytolysin-deficient mutant strain. Testa *et al* (7) have already reported the presence of phospholipase activity in *V. vulnificus*. However, it was a secretory enzyme and could not cause the lysis of erythrocytes.

To identify the role of PLA in the virulence of *V. vulnificus* infection, bacteria-induced cytotoxicity and lethality were studied in the presence of the PLA inhibitor tetracycline. The effect of tetracycline was compared to that of penicillin, another antibiotic with no inhibitory effect on PLA. Both the *V. vulnificus* CVD707-induced cytotoxicity of macrophage and lethality to mice were significantly prevented by tetracycline, but not by penicillin, suggesting that PLA might play an important role in the pathogenesis of *V. vulnificus*

infection. Counts of bacterial numbers in the medium and blood revealed that penicillin had a more potent bactericidal effect than tetracycline. However, their effects on cytotoxicity and lethality were the opposite; tetracycline was much more effective in the prevention of cytotoxicity and lethality. These results suggest that even when alive, *V. vulnificus* is not cytotoxic and lethal when its membranous PLA is inactive. A supply of sufficient iron through PLA-induced hemolysis may facilitate *V. vulnificus* growth and pathogenesis. Inhibition of PLA by tetracycline may be one of the reasons why tetracycline has been used successfully as a drug of choice in *V. vulnificus* infection.

Taken together, our study suggests that *V. vulnificus* PLA plays a pivotal role as a major pathogenic determinant in *V. vulnificus* infection. Compared with phospholipase C or phospholipase D (32,33), little is known about the lethal mechanism of bacterial PLA. Only their potential roles in terms of the disruption of host membranes as well as providing precursors to eicosanoid synthesis are known (34). Better knowledge about the exact mechanism of *V. vulnificus* PLA and its cytosolic cofactor in cytotoxicity and lethality will be helpful in treating *V. vulnificus* infection.

Acknowledgements

This work was supported by a grant from Kye-Nam, Kim Jae-Jung Memorial Fund (to B-H.P.) and by the Regional Research Centers Program of the Korean Ministry of Education and Human Resources Development through the Center for Healthcare Technology Development.

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