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Species-specific hemolysis by *Vibrio vulnificus* cytolysin

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Abbreviations: HU, hemolytic unit; MCV, mean corpuscular volume

Abstract

Cytolysin has been incriminated as one of the important virulence determinants in *V. vulnificus* infection that causes septicemia and serious wound infection. Hemolysis by *V. vulnificus* cytolysin is colloid-osmotic in nature, and cytolysins after binding to membrane oligomerize to form small pores on erythrocyte membrane. The hemolytic sensitivity by *V. vulnificus* cytolysin displayed a great variation between the species. Among the species studied, the half lytic doses of cytolysin for sheep, mouse and human erythrocyte suspensions were 0.40, 0.96 and 2.46 hemolytic units, respectively. There was no significant difference between the species in both the binding and pore formation. Under the hypotonic condition, the stability of erythrocytes from sheep, mouse and human was inversely correlated with the hemolytic susceptibility to the cytolysin. Our results indicate that species difference in hemolytic susceptibility to *V. vulnificus* cytolysin is not dependent on the binding or pore formation, but rather dependent on the unique osmotic stability of erythrocyte membranes, which shows a strong correlation with the size of cells.

Keywords: *Vibrio vulnificus*; cytolysin; hemolysis; species

Introduction

Vibrio vulnificus is an estuarine bacterium that causes septicemia and serious wound infection in humans who are immunocompromised or have underlying diseases such as liver cirrhosis or hemochromatosis (Blake *et al.*, 1979; Park *et al.*, 1991). As one of possible virulence determinants, previous study by Kreger and Lockwood (1981) demonstrated that the cytolysin present in culture medium of *V. vulnificus* showed hemolytic activity and cytotoxicity for mammalian cells in culture, and acted also as a vascular permeability factor.

V. vulnificus cytolysin, produced by most pathogenic strains, is a water-soluble polypeptide of M_r 51,000 (Kim *et al.*, 1992; Miyoshi *et al.*, 1993). Previously, we have reported that hemolysis caused by *V. vulnificus* cytolysin was colloid-osmotic in nature, and that cytolysins bound to membrane were oligomerized to form Ca^{2+} ion-impermeable small pores on the erythrocyte membranes (Kim *et al.*, 1993; Park *et al.*, 1994).

The pathogenetic roles of cytolysin in *V. vulnificus* infection are controversial (Oliver *et al.*, 1986; Wright and Morris, 1991), but cytolysin is very powerful and still is one of the most possible candidates in the pathogenesis of disease (Miyoshi *et al.*, 1993). Even sub-microgram amount of cytolysin is fatal to mice when injected intravenously (Gray and Kreger, 1985; Park *et al.*, 1996). Little is known about the target cell of *V. vulnificus* cytolysin in the body and the relationship between its lethal activity and cytotoxic mechanism involving pore formation.

Studies on the susceptibility of erythrocytes from various animal species for the pore-forming toxins demonstrated a great variability between the species (Cooper *et al.*, 1966; Bernheimer *et al.*, 1975; Kreger, 1984; Cauci *et al.*, 1993). But there were no clear explanations for the underlying mechanism. Qualitative and quantitative receptor differences, surface charge variation or degree of accessibility to key binding sites were thought to be responsible (Cauci *et al.*, 1993). In this report, we have also observed the same hemolytic phenomenon with *V. vulnificus* cytolysin, and the mechanism for the species-difference of hemolytic sensitivity was studied.

Materials and Methods

Bacterial strain and culture

A virulent strain of *Vibrio 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 D.C.). The strain was cultured in heart infusion diffusate broth (Gibco) at 37°C for 4 h as described by Kreger *et al.* (1988).

Purification of cytolysin

The cytolysin was purified to homogeneity from the culture supernatant by ammonium sulfate fractionation, calcium phosphate gel adsorption, quarternary methylamine anion-exchange chromatography and octyl-Sepharose CL-4B chromatography as described by Kim *et al.* (1992).

Assay of hemolytic activity

The hemolytic activity against mouse erythrocytes was determined by the method of Bernheimer and Schwartz (1963). The cytolysin was diluted with phosphate-buffered saline (67 mM Na_2HPO_4, 77 mM NaCl, pH 7.4) containing 1 mg/ml of bovine serum albumin (PBS-BSA). Cytolysin (1 ml) was mixed with the same volume of 0.7% mouse erythrocyte suspensions in PBS-BSA (final 0.35%). The mixture was incubated at 37°C for 30 min and centrifuged. The absorbance of hemoglobin in the supernatant was measured at 545 nm. One hemolytic unit (HU) was defined as that amount which liberates half of the hemoglobin in the erythrocyte suspensions under these conditions. The amount of erythrocytes from other species was adjusted to have the same absorbance at 545 nm with 0.35% mouse erythrocyte suspension after full lysis (A_{545} = 0.9).

Measurement of K^+ release

For the measurement of K^+ release, cytolysin diluted with Tris-buffered saline (20 mM Tris, 140 mM NaCl, pH 7.4) containing 1 mg/ml of bovine serum albumin (1 ml) was mixed with the same volume of 7% erythrocyte suspension in the presence or absence of 50 mM raffinose (Park *et al.*, 1994). The concentration of cytolysin was adjusted to result in approximately 50% lysis of mouse erythrocytes in this condition. After incubation at 37°C for 30 min, the concentration of potassium in the supernatant was determined by flame spectrophotometer (KLiNa Flame, Beckman).

Results

To study the species difference of hemolytic sensitivity by *V. vulnificus* cytolysin, erythrocyte suspensions from various species were incubated with 1 HU of cytolysin at 37°C for 30 min. As shown in Table 1, hemolytic sensitivity by *V. vulnificus* cytolysin displayed a great variation between the species. Among the species studied, sheep erythrocytes were most sensitive, and human and rabbit erythrocytes were most resistant.

To determine the amount of cytolysin which evoke 50% hemolysis, sheep, mouse and human erythrocyte suspensions (containing hemoglobins equivalent to 0.35% mouse erythrocytes) were incubated with various concentrations of cytolysin (Figure 1). The half lytic doses of cytolysin for sheep, mouse and human erythrocyte suspensions were 0.40, 0.96 and 2.46 HU,

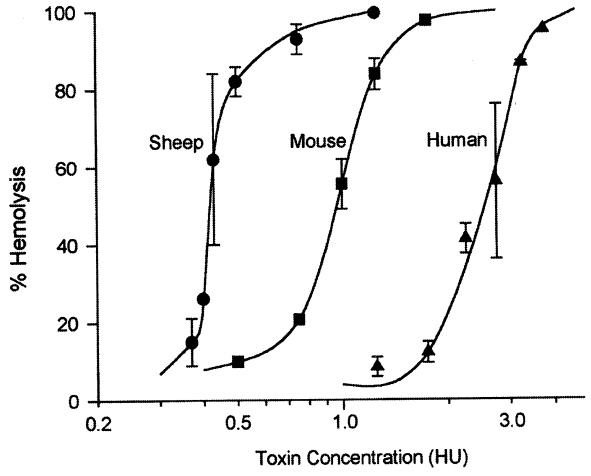

Figure 1. Concentration-dependent hemolysis of sheep, mouse and human erythrocytes by *V. vulnificus* cytolysin. Various concentrations of cytolysin (1 ml) were mixed with the same volume of 0.7% mouse erythrocyte suspensions in PBS-BSA. Erythrocytes from sheep or human were adjusted to have the same absorbance after full lysis with the standard 0.35% mouse erythrocyte suspensions. After incubation at 37°C for 30 min and brief centrifugation, the released hemoglobin in the supernatant was measured at 545 nm. Each point represents the mean ± S.D. of at least three separate experiments.

Table 1. Sensitivity of erythrocytes from various animal species to cytolysin produced by V. vulnificus. Erythrocyte suspensions having the same hemoglobin contents with 0.35% mouse erythrocytes were incubated with 1 HU of cytolysin at 37(C for 30 min. The released hemoglobin was measured by reading absorbance at 545 nm. The results were expressed as mean (S.D. of three separate experiments.

Species	A_{545nm}
Sheep	0.78 ± 0.02
Mouse	0.45 ± 0.11
Rat	0.43 ± 0.09
Cow	0.37 ± 0.10
Dog	0.12 ± 0.04
Rabbit	0.02 ± 0.01
Human	0.02 ± 0.00

respectively. Rat erythrocytes showed similar sensitivity to those of mouse (data not shown).

Hemolysis by pore-forming toxins is known to occur through the three sequential steps: (i) binding of toxins to the membrane, (ii) formation of oligomer resulting in the leakage of small ions, and finally (iii) lysis of cells (Bhakdi and Tranum-Jensen, 1988; Bhakdi and Tranum-Jensen, 1991; Kim *et al.*, 1993). To determine whether the binding of cytolysin to erythrocyte membrane was different between species, 0.35% erythrocyte suspensions of sheep, mouse and human were incubated with 10 HU of cytolysin at 4°C. The binding of *V. vulnificus* cytolysin is shown to be temperature-independent and the bound cytolysins remain as monomer at 4°C on the erythrocyte membranes (Kim *et al.*, 1993). After brief centrifugation, the remaining hemolytic activity of unbound cytolysin in the supernatant was assayed using mouse erythrocytes (Figure 2). Cytolysin was bound to erythrocyte membranes within 1 min, and there were no significant differences in the binding between the species.

When the cells were incubated with cytolysin at 37°C, hemolysis occurred and nearly all the intracellular K^+ was found in the supernatant (Figure 3). Raffinose is known to prevent the cytolysin-induced hemolysis at the cell lysis step as an osmotic protectant, without affecting the small ion release (Kim *et al.*, 1993). When the cells were incubated with cytolysin in the presence of 50 mM raffinose, hemolysis was inhibited in all the three species, but the K^+ release was not affected (Figure 3). These results indicated that species difference of hemolytic sensitivity was not due to the binding or oligomerization of cytolysins, and that it was exerted after pore formation.

We checked the resistance of erythrocytes from three species under the hypotonic milieu (Figure 4). Human erythrocytes were most resistant and sheep cells were most sensitive under the hypotonic stress, while the mouse cells were in the middle. The calculated half lytic concentrations of NaCl for sheep, mouse and human erythrocytes were 0.57, 0.47 and 0.40%, respectively.

Discussion

Erythrocytes have been widely used as a simple cell model to study the cytotoxic mechanism of cytolysins. Hemolysis caused by bacterial cytolysins may be of two types; colloid-osmotic hemolysis by staphylococcal α-toxin (Fussle *et al.*, 1981) or *E. coli* hemolysin (Bhakdi *et al.*, 1986), and noncolloid-osmotic hemolysis by thiol-activated hemolysins such as streptolysin O (Buckingham and Duncan, 1983). We already reported that

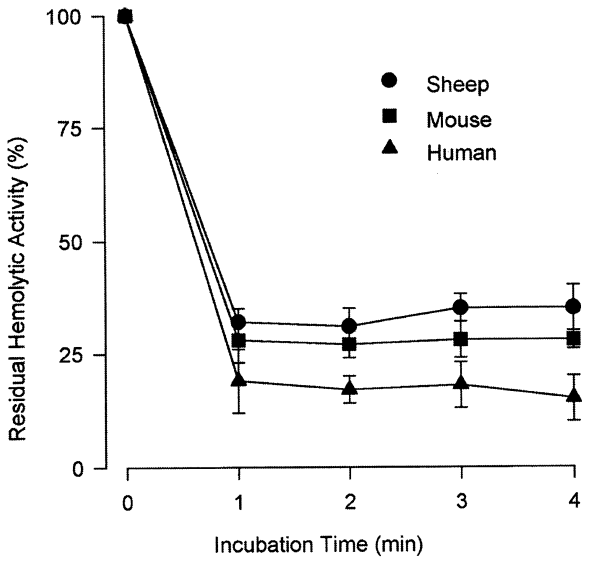

Figure 2. Binding of *V. vulnificus* cytolysin to sheep, mouse and human erythrocytes. Erythrocyte suspensions (equivalent to 0.35% mouse erythrocytes) were incubated with 10 HU of cytolysin at 4°C. After brief centrifugation, hemolytic activity of unbound cytolysin in the supernatant was assayed using mouse erythrocytes. Each point represents the mean ± S.D. of three separate experiments.

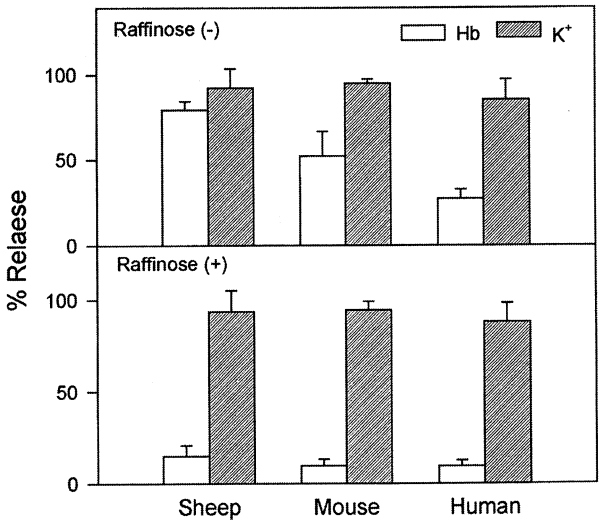

Figure 3. Hemoglobin and K^+ release in the presence or absence of osmotic protectant. Cytolysin (1 ml) was mixed with the same volume of 7% erythrocyte suspension in the presence or absence of 50 mM raffinose. The concentration of cytolysin was adjusted to result in approximately 50% lysis of mouse erythrocytes in this condition. After incubation at 37°C for 30 min, released hemoglobin was measured by absorbance at 545 nm and released potassium in the supernatant was determined by flame spectrophotometer. The results were expressed as mean ± S.D. of three separate experiments.

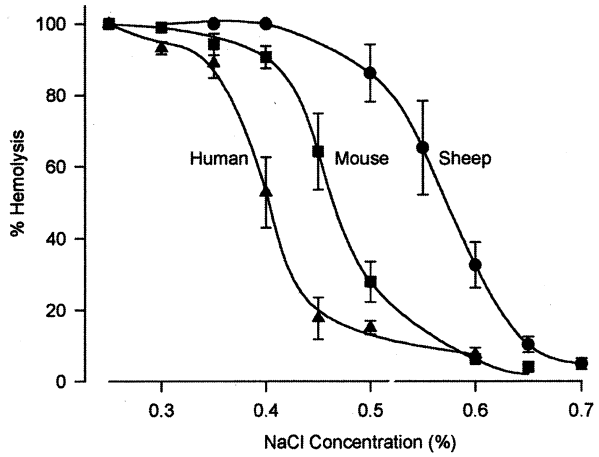

Figure 4. Stability of human, mouse and sheep erythrocytes under the hypotonic milieu. Erythrocytes of sheep, mouse and human (equivalent to 0.35% mouse erythrocytes) were suspended with various concentrations of NaCl solutions. After incubation at 37°C for 30 min, released hemoglobin was measured by absorbance at 545 nm. Each point represents the mean ± S.D. of at least three separate experiments.

hemolysis caused by *V. vulnificus* cytolysin was colloid-osmotic in nature and that cytolysins, after binding to membrane, were oligomerized to form small pores on the erythrocyte membranes (Kim *et al.*, 1993).

The hemolytic sensitivity by pore-forming cytolysins displayed a great variation between the species (Cooper *et al.*, 1966; Bernheimer *et al.*, 1975; Kreger, 1984; Cauci *et al.*, 1993). The order of hemolytic sensitivity was also different from toxin to toxin (Bhakdi and Tranum-Jensen, 1988). The basis for species specificity of bacterial hemolysins is still unknown. For the cytolysin of *Vibrio damsela* (Kreger, 1984) or *Gardnerella vaginalis* (Cauci *et al.*, 1993), the difference in toxin binding was suspected as a cause. Little is known about the membrane receptor for *V. vulnificus* cytolysin. Free cholesterol has been thought as a candidate based on the fact that it inhibits the hemolytic activity of cytolysin (Kim *et al.*,1993).

Hemolytic sensitivity to purified *V. vulnificus* cytolysin also showed species difference. Among the species studied, we chose sheep, mouse and human erythrocytes, according to the increasing resistance to hemolytic action of cytolysin, and clarified the underlying mechanism for the species-specific hemolytic susceptibility by studying the binding, pore formation and cell lysis steps.

The binding of *V. vulnificus* cytolysin to mouse erythrocytes shows similar patterns at 0°C and 37°C; the binding process is thus temperature-independent. But the cell disruption is temperature-dependent process and invariably accompanied by oligomerization of cytolysin (Kim *et al.*, 1993). Erythrocytes from all the species studied showed rapid and temperature-independent binding, and temperature-dependent cell disruption. There was no significant difference between the species on the binding of cytolysin.

The size of pores produced by bacterial pore-forming toxins was variable from 30-35 nm of streptolysin O (Buckingham and Duncan, 1983) to 2-3 nm of staphylococcal alpha-toxin (Fussle *et al.*, 1981) or *E. coli* hemolysin (Bhakdi *et al.*, 1986). Pores formed by *V. vulnificus* cytolysin have effective diameter of about 1 nm, impermeable to raffinose but freely permeable to small ions like K^+ (Kim *et al.*, 1993). A study of the comparative release of K^+ and hemoglobin was undertaken to elucidate the species difference of pore-formation in the presence or absence of raffinose, an osmotic protectant, at a 50 mM concentration for balancing the osmotic pressure generated by hemoglobin (Kasschau and Prill, 1988). There was no species difference in K^+ release in the presence or absence of raffinose, while hemolysis was prevented by raffinose. The fact that nearly all the intracellular potassium was released by the cytolysin suggests that enough number of pores for hemolysis were already formed on the cell membranes. These results indicate that species difference on hemolytic sensitivity was due to the difference in cell lysis step rather than its binding or pore formation.

Under the hypotonic state, the stability of erythrocytes was different from species to species. The stability of erythrocytes was decreased from human, to mouse, and to sheep in order, exactly reverse to the susceptibility of hemolysis by cytolysin, suggesting that different hemolytic susceptibility depends on the osmotic stability of erythrocyte membranes.

What makes these differences in membrane stability between the species requires further studies. One of the interesting points is the relationship with the size of erythrocytes. The more susceptible to cytolysin or osmotic stress, the smaller the cell size is. The mean corpuscular volume (MCV) of human, mouse and sheep erythrocytes were 82.0 ~ 92.0, 31.0 ~ 62.0 and 28.0 ~ 34.9 μ^3, respectively (Mitruka and Rawnsley, 1981). The half lytic dose for rat erythrocytes were nearly same with mouse, and the MCV for rat erythrocytes were 44.5 ~ 69.0 μ^3. The correlation coefficient between the half-lytic dose of cytolysin and MCV in human, rat, mouse and sheep erythrocytes was 0.956.

Based on our observations, we can conclude that species difference in hemolytic susceptibility is not dependent on the binding or pore formation, but rather dependent on the unique osmotic stability of erythrocyte membranes, which has a strong correlation with the size of cells.

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