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Enhancement of *Vibrio parahaemolyticus* Virulence by Lysed Erythrocyte Factor and Iron

I. KARUNASAGAR,† SAM W. JOSEPH, ROBERT M. TWEDT,‡ HOWARD HADA, AND RITA R. COLWELL*

Department of Microbiology, University of Maryland, College Park, Maryland 20742

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The effect of lysed blood on the virulence of *Vibrio parahaemolyticus* for mice was investigated. A factor present in lysed erythrocytes was found to greatly reduce the 50% lethal dose of *V. parahaemolyticus* for mice. Similar effects were observed with ferric ammonium citrate and manganous sulfate. *V. parahaemolyticus* grown in brain heart infusion containing lysed erythrocyte factor appeared to produce a lethal toxin which was either inapparent or absent when the organism was grown in brain heart infusion alone.

Despite extensive studies on the Kanagawa hemolysin of *Vibrio parahaemolyticus*, its role in pathogenesis remains to be clarified. A hemolysin produced by *V. parahaemolyticus* has been purified and studied by several groups of workers (4, 5, 9–11, 15, 16). Although the purified hemolysin has been reported to give a positive reaction in the rabbit ileal loop test, the high concentrations required (200 μg) to produce an effect, compared with choleragen (2 μg), raise the question of whether *V. parahaemolyticus* hemolysin is enteropathogenic (5). On the basis of studies of the ileal loop effective dose of Kanagawa-positive (KP^+) *V. parahaemolyticus*, Twedt et al. (13) suggested that pathogenicity of KP^+ strains may involve participation of virulence mechanisms in addition to the Kanagawa hemolysin.

In evaluating the role(s) of hemolysin in the pathogenesis of *V. parahaemolyticus*, the observation that the availability of iron in the body of an infected host is crucial in determining the ability of bacterial species to proliferate in tissues and body fluids (2, 14) led us to consider a similar or related factor in *V. parahaemolyticus* pathogenesis. Bornside et al. (1) have suggested that hemoglobin acts to enhance bacterial growth in vivo, important in cases in which hemolysis and infection coexist. Studies by Linggood and Ingram (8) indicated that the Hly plasmid of hemolytic strains of *Escherichia coli* acts as a virulence factor by enabling the bacteria to obtain iron for growth from lysed erythrocytes of infected animals. Thus, in this context, the role of lysed blood on the virulence of *V. parahaemolyticus* to mice was examined. Results reported here indicate that a factor is present in lysed erythrocytes which enhances the virulence of *V. parahaemolyticus*.

MATERIALS AND METHODS

Bacterial strains. *V. parahaemolyticus* 116-78, a Kanagawa-positive strain belonging to serotype O5:K17, was isolated from stool, and 1308-77, a Kanagawa-negative strain of the same serotype, was isolated from seafood. Both cultures were maintained at room temperature on tryptic soy agar (Difco Laboratories, Detroit, Mich.) containing 3% NaCl.

Mouse virulence tests. Stock cultures were transferred twice in brain heart infusion (BHI) broth (Difco) containing 1% NaCl before inoculation into 50 ml of the same medium before virulence testing. Cultures incubated at 37°C for 12 h were used in all experiments. Samples (1 ml) of serial 10-fold dilutions of a test preparation in sterile, physiological buffered saline were inoculated intraperitoneally into 25-day-old adult NIH/NMRI white mice. The mice were observed at hourly intervals during the first 6 h and then at 24 and 48 h. Peritoneal fluids of both morbid and surviving mice were cultured on thiosulfate-citrate-bile-salts-sucrose agar (Oxoid U.S.A. Inc., Columbia, Md.) to recover injected strains.

Lysed erythrocyte components. Blood from New Zealand white rabbits was used throughout the experiment. Lysed blood was prepared by adding 2 volumes of sterile distilled water to citrated whole blood and shaking it in a vortex mixer. To study the effect of individual blood components, the citrated whole blood was centrifuged to separate plasma from erythrocytes. The erythrocytes were washed twice in sterile saline and added to a culture of *V. parahaemolyticus* at a final concentration of 5% before injection. Lysed erythrocyte extract (LEE) was prepared by adding 5 volumes of sterile distilled water to a pellet of washed erythrocytes, with thorough mixing, followed by centrifugation to separate the stroma. The clear supernatant was stored at 4°C until used. Plasma and LEE were added to equal volumes of *V. parahaemolyticus* cultures before injection.

Metal ions. Ferric ammonium citrate (Fisher Scientific Co., Pittsburgh, Pa.), manganous sulfate (J. T. Baker Chemical Co., Phillipsburg, N.J.), and magnesium sulfate (Baker) were each dissolved in distilled water to give 200 μg of metal ions per ml. The iron salt solution was sterilized by filtration through membrane filter units (0.45-μm pore size; Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.), and the other two were autoclaved at 121°C for 15 min. Solutions were mixed with equal volumes of test culture so that 1 ml of mixture injected per mouse contained 100 μg of the selected metal ion.

Other chemicals. Hemin (Sigma Chemical Co., St. Louis, Mo.) was added to cultures at a final concentration of 0.40 mg/ml. Desferal (500 mg; desferrioxamine mesylate; Ciba-Geigy Corp., Summit, N.J.) was suspended in 2 ml of sterile saline and aseptically added to 8 ml of LEE (3). The mixture was shaken on a reciprocating shaker for 24 h at 100 strokes per min at room temperature, and equal volumes of culture and LEE-Desferal mixture were prepared before inoculating 1.0 ml into mice.

Virulence of washed cells and culture filtrates. *V. parahaemolyticus* 1308-77 was grown in BHI (3% NaCl) in the presence or absence of 5% LEE or 5% erythrocytes for 12 h

* Corresponding author.
† Present address: College of Fisheries, University of Agricultural Sciences, Mangalore-575002, Karnataka, India.
‡ Present address: Food Microbiology Branch, Food and Drug Administration, Cincinnati, OH 45226.

TABLE 1. Effect of lysed rabbit erythrocytes on mouse lethality of *V. parahaemolyticus* strains

Strain no.	No. of cells injected	Additive	No. of mice dead/ no. tested
1308-77	3.0×10^8	Saline[a]	1/8
	3.0×10^7	Saline	0/8
	3.0×10^6	Saline	0/8
	3.0×10^5	Saline	0/8
	3.0×10^8	LE[b]	6/7
	3.0×10^7	LE	8/8
	3.0×10^6	LE	2/8
	3.0×10^5	LE	0/8
116-78	6.0×10^7	Saline[c]	2/7
	6.0×10^6	Saline	0/8
	6.0×10^5	Saline	0/8
	6.0×10^7	LE[d]	5/8
	6.0×10^6	LE	0/8
	6.0×10^5	LE	0/8
Medium only	0	LE	0/8

[a] Approximate $LD_{50} = 3 \times 10^8$ cells.
[b] Approximate $LD_{50} = 6 \times 10^6$ cells. LE, Lysed erythrocytes.
[c] Approximate $LD_{50} = 6 \times 10^7$ cells.
[d] Approximate $LD_{50} = 4 \times 10^7$ cells.

and centrifuged, and the supernatant was filtered through a membrane filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.) and designated cell-free culture filtrate. The cell pellets were washed in sterile saline twice and finally resuspended to their original culture volume in sterile saline. The effect of washed cells or cell-free culture filtrate on mice was studied by injecting intraperitoneally 1 ml of culture filtrate or 10-fold dilutions of washed cells.

RESULTS

V. parahaemolyticus 1308-77, a Kanagawa-negative strain, was not dramatically effective in killing mice in the absence of lysed blood, even at a concentration of 3.0×10^8 cells, whereas addition of lysed blood to the bacterial culture increased virulence significantly (Table 1). Interestingly, the lethal dose of Kanagawa-positive strain 116-78 was not enhanced significantly by the addition of lysed blood. Among the blood components studied, LEE showed the greatest virulence-enhancing effect, followed by whole erythrocytes (Table 2). Plasma and hemin did not show a significant effect on virulence. From these results, it was hypothesized that LEE may provide iron, which is required by the bacteria for proliferation after intraperitoneal injection. When selected metal ions were tested for their effect on virulence of *V. parahaemolyticus*, iron acted similarly to LEE (Table 3). Addition of manganese revealed a virulence-enhancing effect, whereas addition of magnesium did not. Addition of metal ions to the medium only did not result in toxicity for mice at the concentrations used (100 μg).

Desferal, commonly used for treatment of pathological iron deposition, specifically chelates iron from transferrin (3). Its effect on virulence enhancement by LEE was examined as follows. If the virulence enhancement factor in LEE was iron alone, treatment of LEE with Desferal should reduce the virulence-enhancing effect. Indeed, Desferal, at a concentration of 50 mg/ml, counteracted the virulence-enhancing effect of LEE on *V. parahaemolyticus* injected into mice at a concentration of 10^6 cells, with higher concentrations of Desferal toxic for mice (Table 4).

TABLE 2. Effect of blood components on virulence of *V. parahaemolyticus* 1308-77 for mice

No. of cells injected	Additive	No. of mice dead/ no. tested
1.3×10^8	None[a]	6/6
1.3×10^8	Plasma[b]	5/6
1.3×10^8	Erythrocytes (5%)[c]	6/6
1.3×10^8	LEE[d]	6/6
1.3×10^8	Hemin[e]	5/6
1.3×10^7	None	0/6
1.3×10^7	Plasma	0/6
1.3×10^7	Erythrocytes (5%)	6/6
1.3×10^7	LEE	6/6
1.3×10^7	Hemin	0/6
1.3×10^6	None	0/6
1.3×10^6	Plasma	0/6
1.3×10^6	Erythrocytes (5%)	2/6
1.3×10^6	LEE	6/6
1.3×10^5	None	0/6
1.3×10^5	Plasma	0/6
1.3×10^5	Erythrocytes (5%)	0/6
1.3×10^5	LEE	1/6
None	Plasma	0/6
None	Erythrocytes (5%)	0/6
None	LEE	0/6
None	Hemin	0/6

[a] Approximate $LD_{50} = 6.3 \times 10^7$ cells.
[b] Approximate $LD_{50} = 7.3 \times 10^7$ cells.
[c] Approximate $LD_{50} = 3.8 \times 10^6$ cells.
[d] Approximate $LD_{50} = 5.2 \times 10^5$ cells.
[e] Approximate $LD_{50} = 7.3 \times 10^7$ cells.

Further studies were conducted to determine whether the virulence-enhancing effect of LEE was related to a factor in

TABLE 3. Effect of metal ions on virulence of *V. parahaemolyticus* 1308-77 for mice

No. of cells injected	Metal ion (100 μg/ml)	No. of mice dead/ no. tested
7.4×10^7	None added	0/6
7.4×10^6	None added	0/6
7.4×10^5	None added	0/6
7.4×10^7	Fe^{3+}[a]	6/6
7.4×10^6	Fe^{3+}	1/6
7.4×10^5	Fe^{3+}	0/6
7.4×10^7	Mn^{2+}[b]	4/6
7.4×10^6	Mn^{2+}	0/6
7.4×10^5	Mn^{2+}	0/6
7.4×10^7	Mg^{2+}	0/6
7.4×10^6	Mg^{2+}	0/6
7.4×10^5	Mg^{2+}	0/6
None	Fe^{3+}	0/6
None	Mn^{2+}	0/6
None	Mg^{2+}	0/6

[a] Approximate $LD_{50} = 1.4 \times 10^7$ cells.
[b] Approximate $LD_{50} = 1.5 \times 10^7$ cells.

TABLE 4. Effect of Desferal on virulence of LEE-enhanced *V. parahaemolyticus* 1308-77

No. of cells injected	Desferal (50 mg/ml)	LEE	No. of mice dead/ no. tested
1.3×10^8	None	None	6/6
1.3×10^8	None	+	6/6
1.3×10^8	+	+	6/6
1.3×10^7	None	None	0/6
1.3×10^7	None	+	6/6
1.3×10^7	+	+	4/6
1.3×10^6	None	None	0/6
1.3×10^6	None	+	6/6
1.3×10^6	+	+	3/6

V. parahaemolyticus cells grown in the presence of LEE or to a metabolite, enzyme, or toxin, the release of which was triggered by a factor present in LEE. Thus, virulence studies were conducted with washed cells. Washed cells were not lethal to mice, even at a concentration of 7.5×10^7 cells, irrespective of whether they were grown in BHI alone or in BHI containing 5% LEE or erythrocytes (Table 5). It must be pointed out that when LEE was added to BHI-grown cells before injection, lethality occurred with 2 logs fewer cells (Table 2). Interestingly, a filtrate of *V. parahaemolyticus* culture grown in BHI containing 5% LEE was highly lethal to mice, whereas BHI- and BHI plus erythrocyte-grown culture filtrates were not lethal (Table 5).

DISCUSSION

It appears from the results obtained in this study that a factor present in lysed blood cells (lysed erythrocyte factor) enhances the virulence of *V. parahaemolyticus*. It is plausible that iron in LEE is the major contributing factor for the enhancement of virulence. A virulence-enhancing effect of iron has been demonstrated for a number of gram-positive as well as gram-negative bacteria (14). In the case of *Neisseria meningitidis* and *Vibrio cholerae*, mucin acts to enhance virulence, an effect partially attributed to the iron content of mucin (3, 6). The absence of iron appears to be a limiting factor for the growth of *V. parahaemolyticus* in serum. When *V. parahaemolyticus* is injected into mice, iron limitation in the intraperitoneal environment can prevent bacterial proliferation, giving sufficient time for host defenses to clear invading bacteria. This is supported by our observation that peritoneal fluids of surviving mice in this study were sterile. When iron is provided, in the form of ferric ammonium citrate, the bacteria can proliferate, attaining lethal concentrations of cells. The 50% lethal dose (LD_{50}) in the presence of LEE was less than in the presence of ferric ammonium citrate, which is probably attributable to the amount and ready availability of iron from LEE. Calvin et al. (3) attributed the more predominant, virulence-enhancing effect of iron dextran (molecular weight, 180,000) on *N. meningitidis*, compared with iron sorbitol citrate (molecular weight, 5,000), to the difference in the size of the molecule.

Results obtained with hemin were puzzling. The low solubility of hemin under physiological conditions most likely rendered the bound iron unavailable to the bacteria. Interestingly, manganese stimulated the effect of Fe^{3+}. In the case of *Listeria monocytogenes* (12) and *E. coli* (8), this phenomenon has been attributed to replacement of iron by manganese in the transferrin (12).

Since heat-labile hemolysins have not been correlated with virulence in *V. parahaemolyticus*, their effectiveness in vivo, as an iron-releasing mechanism, provides a fascinating hypothesis which must be verified. Nevertheless, Kanagawa hemolysin has been associated with the virulence of *V. parahaemolyticus*, an important function of which now appears to be the rendering of available iron needed for growth and metabolism of this organism. This may explain, in part, its presence in 95 to 99% of the *V. parahaemolyticus* strains recovered from clinical cases. The most appealing conclusion to be drawn from these studies is the selective advantage provided hemolysin-producing (KP^+) cells. This is, perhaps, a partial answer to the provocative question as to why KP^+ cells are primarily isolated from patients and KP^- cells are primarily isolated from environmental samples, heretofore one of the major enigmas of the *V. parahaemolyticus* infectious mechanism complex.

It remains to be proven whether the role of LEE in virulence of *V. parahaemolyticus* is only to provide iron for growth and metabolism or whether production of toxin is also stimulated. Results shown in Table 5 suggest that KP^- *V. parahaemolyticus* grown in BHI amended with LEE produces a lethal factor not produced in BHI alone or in the presence of erythrocytes. Because KP^- *V. parahaemolyticus* is nonhemolytic, the factor available in LEE most likely cannot be released from erythrocytes in BHI. The role of Fe^{3+} in the production of a presumed toxin should be investigated. Karunasagar et al. (7) have already shown the importance of iron in synthesis of vascular permeability factor by *V. cholerae*. Furthermore, the nature of a presumed toxin and its role, if any, in pathogenesis by *V. parahaemolyticus* grown in BHI containing LEE, compared with the same organism grown in BHI but injected concomitantly with LEE, suggests that a surface-associated virulence factor is not produced by cells grown in the presence of LEE.

We conclude from the results of this study that KP^+ cells have a clear advantage over KP^- *V. parahaemolyticus* during the infectious process and that the hemolysin of KP^+ cells could provide the iron required for growth of the organism. LEE produced in vivo by the action of hemolysin may, indeed, also stimulate production of toxins or other metabolites by the bacteria.

TABLE 5. Virulence of washed cells of *V. parahaemolyticus* 1308-77 previously grown in BHI in the presence or absence of erythrocytes and LEE

Growth medium	No. of cells injected	No. mice dead/ no. tested
BHI	0 (CF)[a]	0/6
BHI	7.5×10^7	0/6
BHI	7.5×10^6	0/6
BHI	7.5×10^5	0/6
BHI + LEE	0 (CF)	6/6
BHI + LEE	7.5×10^7	0/6
BHI + LEE	7.5×10^6	0/6
BHI + LEE	7.5×10^5	0/6
BHI + RBC[b]	0 (CF)	0/6
BHI + RBC	7.5×10^7	0/6
BHI + RBC	7.5×10^6	0/6
BHI + RBC	7.5×10^5	0/6

[a] CF, Culture filtrate.
[b] RBC, Erythrocytes.

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