Edema and Hemoconcentration in Mice Experimentally Infected with *Vibrio vulnificus*

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*Vibrio vulnificus* (lactose-positive *Vibrio*), a recently recognized pathogenic marine species, produced extreme hemoconcentration and death within 3 to 6 h after subcutaneous or intraperitoneal injection of $10^8$ viable cells into mice; hematocrit values approached 70% (normal, 45%). About 1 ml of edema fluid accumulated at the site of each subcutaneous injection, and locally increased vascular permeability was demonstrated by a skin bluing assay, using Evans blue dye. A corresponding fluid accumulation did not occur in the peritoneal cavity after an intraperitoneal injection. Filter-sterilized supernatants of cultures grown under a variety of conditions did not produce local edema or lethality, nor did whole *Vibrio* cells killed by a variety of methods or disrupted by sonic oscillation. Edema fluids collected from infected mice and sterilized by filtration had no effect when they were injected subcutaneously or intraperitoneally into mice. Inocula of $10^8$ viable cells of *V. vulnificus* contained within a diffusion chamber implanted subcutaneously did not produce skin bluing, edema, or lethality; *Vibrio* cells remained viable and virulent within these chambers for at least 2 weeks. These experiments suggested that vascular permeability changes in *V. vulnificus* infections may not be attributable to a diffusible toxin and may require direct contact between host cells and viable *Vibrio* cells.

*Vibrio vulnificus* is a marine *Vibrio* species recently recognized as a human pathogen (3, 10). This organism is phenotypically very similar to *Vibrio parahaemolyticus* and *Vibrio alginolyticus* but may be differentiated from those two species by a number of physiological characteristics (3). Hollis et al. (10) differentiated clinical isolates of *V. vulnificus* from closely related species by the ability of the isolates to hydrolyze o-nitrophenyl-β-D-galactopyranoside and to ferment lactose. This organism has been referred to as the lactose-positive *Vibrio* and as *Beneckea vulnifica*; however, Baumann et al. (2) have concluded that the genus *Beneckea* should be abolished. Accordingly, we use the name *V. vulnificus*, as suggested by Farmer (8).

Deoxyribonucleic acid hybridization studies (6, 13) have shown that *V. vulnificus* strains are highly related to each other and distinct from other *Vibrio* species, including *V. parahaemolyticus* and *V. alginolyticus*. Unlike the latter two species, *V. vulnificus* produces rapidly progressive wound infections and frequently is isolated from the blood of patients. The clinical and epidemiological features of 39 cases of *V. vulnificus* infection have been reviewed by Blake et al. (4). Two clinical forms of the disease are recognized. One is fulminating septicemia, in which *V. vulnificus* is isolated from blood cultures and sometimes also from secondary skin lesions. The apparent portal of entry is the gastrointestinal tract, and an association has been made with consumption of raw oysters. This form of infection occurs primarily in patients with preexisting hepatic disease; 4 of 24 such patients had hemochromatosis. Severe hypotension and shock may result from this form, and 11 of 24 recorded cases were fatal, often within 2 or 3 days of onset of disease. The other form of *V. vulnificus* infection is a rapidly progressive cellulitis resulting from infection of seawater-associated wounds, such as those sustained while cleaning crabs or digging clams. This form occurs in apparently healthy people, is characterized by marked edema and necrosis usually requiring incision and drainage or debridement as well as antibiotic therapy, and sometimes results in septicemia. Unlike *V. parahaemolyticus*, *V. vulnificus* does not appear to produce gastroenteritis.

Experimental animal infections with *V. vulnificus* were studied by Poole and Oliver (12), who reported that subcutaneous (s.c.) injection of $10^6$ colony-forming units (CFU) into Institute of Cancer Research strain mice produced severe local edema, bacteremia, and death within 3 to 6 h. The 50% lethal dose of *V. vulnificus* for
mice was about $10^6$ CFU/mouse when the organism was injected s.c.; surviving animals developed necrotic lesions at the injection site. In contrast, *V. parahaemolyticus* produced no edema or mortality when injected s.c., even when $10^6$ cells per mouse were injected. Attempts to demonstrate fluid accumulation by injection of *V. vulnificus* into ligated ileal loops in rats and rabbits resulted instead in rapid development of septicemia and death of the animals.

We have observed that extreme hemoconcentration accompanies edema in mice infected with *V. vulnificus* and undertook the experiments described here to investigate whether an extracellular toxin might be the basis for the massive loss of intravascular fluid which characterizes this type of infection in the mouse model. Although this study verified hemoconcentration and edema in mice injected with *V. vulnificus*, we were not able to demonstrate the presence of a toxin responsible for these effects.

**MATERIALS AND METHODS**

**Cultures.** Centers for Disease Control strains B3547 and C7184 were provided by R. E. Weaver and D. G. Hollis. Strain C7184 is a human blood isolate; strain B3547 was isolated originally from the gangrenous leg of a septic patient described by Roland (14), *D. G.* The latter strain was included in previous taxonomic (3) and deoxyribonucleic acid-deoxyribonucleic acid reassociation (6, 13) studies; both of these strains were used by Poole and Oliver (12) in their experimental infections.

**Media and inocula.** Cultures were stored frozen in defibrinated rabbit blood which was maintained under liquid nitrogen. Working stocks were maintained at room temperature on modified salt water yeast extract medium slants, as previously described (12). The *Vibrio* cells used for injection into mice were grown in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with an additional 2.5% NaCl and were incubated for 4 to 6 h on a rotary shaker at 35°C or statically at room temperature overnight. Other experimental media and conditions of incubation were as indicated below. *Vibrio* cells were harvested by centrifugation at 10,000 × g for 15 min and then suspended in sterile 0.85% NaCl. The inoculum size was standardized by turbidimetry with a Bausch & Lomb Spectronic 20 colorimeter and was confirmed for each experiment by determining the total viable counts, in duplicate, on modified salt water yeast extract agar or on brain heart infusion agar supplemented with an additional 2.5% NaCl. The inoculum volume was 0.1 ml for both s.c. and intraperitoneal (i.p.) injections.

Casein hydrolysate salts medium contained the following (in grams per liter): vitamin-free, salt-free, acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio), 1.0; KCl, 0.75; and MgSO$_4$·7H$_2$O, 7.0. This medium was prepared in distilled water and adjusted to pH 7.3. After autoclaving, other components to be tested were added aseptically.

**Animals.** The mice used were Institute of Cancer Research strain females which were 6 to 14 weeks old and weighed 25 to 34 g. They were obtained from Charles River Breeding Laboratories, Wilmington, Mass., or from Perfection Breeders, Douglassville, Pa. Mice were allowed food and water ad libitum at all times.

**Visualization of local edema.** The lumbar area of the back of each mouse was depepilated with Nair (Carter Products, New York, N.Y.), washed thoroughly with tap water, and allowed to dry. Immediately before s.c. injection of *Vibrio* cells or of saline, these mice were injected i.p. with 0.5 ml of Evans blue (0.5% aqueous solution), which enters the vascular system and binds to albumin (15). The Evans blue method was used to facilitate visual detection of the edematous area produced by injection of live *Vibrio* cells. This method was also used in attempts to produce edema by injection of culture filtrates, products, or extracts of *V. vulnificus*.

**Hematocrit determination.** Blood samples were collected from mice by retroorbital puncture with heparinized microhematocrit tubes (16 μl; Propper Manufacturing Co., Long Island City, N.Y.). Samples were centrifuged for 5 min with a microhematocrit centrifuge (International Equipment Co., Div. of Damon Corp., Needham Heights, Mass.), and the hematocrit values were determined.

**RESULTS AND DISCUSSION**

**Hematocrit changes.** Mice which had been injected with *V. vulnificus* either i.p. or s.c. developed apparent hemoconcentration during the following 3 to 6 h. The blood became increasingly viscous and difficult to obtain for sampling. Figure 1 shows representative increases in hematocrit values for four mice after i.p. injection of $2 \times 10^7$ CFU of *V. vulnificus* per mouse compared with values observed in a control mouse injected with saline. Hematocrit values increased within 4 h after *Vibrio* injection from a normal value of 44 to 46% to values approaching 70%. This phenomenon was observed in more than 50 mice, using both experimental strains of *V. vulnificus*. As indicated on Fig. 1, all four experimental animals died within 4.5 h after injection. Similar increases in hematocrit values were obtained with s.c. inocula of comparable size, but the time course was extended to about 6 h.

**Detection and quantification of edema.** Mice which had been injected i.p. with Evans blue developed a zone of marked bluing at the site of s.c. injection of *V. vulnificus*. The blue zone coincided with the area of palpable edema and developed into a massive swelling during the 4 h after injection of *Vibrio* cells. Skin bluing was not observed in control mice injected with...
saline instead of Vibrio cells. Distribution of the dye into the extravascular fluid was slow in uninfected mice, which began to show generalized cutaneous bluing only after 18 to 24 h.

To quantify the amount of fluid accumulation, 10 mice were sacrificed 4 h after s.c. injection of 10⁸ V. vulnificus cells. A standard area of full-thickness skin surrounding the edematous site was excised and weighed. By subtracting the mean mass of similar skin samples from 12 control mice which had been injected with saline, we determined that the mean mass of edema fluid accumulating per infected mouse was 951 ± 81 mg. The presence of about 1 ml of edema fluid represented approximately two-thirds of the estimated total plasma volume of a mouse of that size (17).

Mice injected i.p. did not have noticeable collections of fluid in their peritoneal cavities despite the large increases in hematocrit values observed. The fluid lost from the vascular system was not excreted, as urine output was not observed when mice were maintained on filter paper and body weight did not change. These results suggested that fluid loss into some or all tissues must have occurred after i.p. injection. A histological investigation of the basis for this increased vascular permeability is currently in progress.

Testing of nonviable materials. The massive edema observed after s.c. injection and the hemoconcentration which developed after s.c. or i.p. injection suggested the possible involvement of a toxin that acted to increase vascular permeability. Therefore, we tested broth supernatants, killed cells, and freshly disrupted cells of V. vulnificus for the ability to produce edema or mortality when injected into mice (Table 1). No preparation other than viable bacteria had any effect on the mice.

Three different filter materials were used for sterilization of supernatants to minimize the possibility that a toxin might have been removed by adsorption of the filters (Table 1).

Many marine Vibrio species produce inducible collagenases (11), and such enzymes were considered as possible candidates for an edema-producing toxin. Various nutritional manipulations were suggested by this possibility, as well as by the conditions found optimal (9) for enterotoxin production in Escherichia coli. Gilligan and Robertson reported that heat-stable enterotoxin was not produced in the presence of glucose, whereas heat-labile enterotoxin was pro-

![Figure 1](http://iai.asm.org/)  
**FIG. 1.** Effect of V. vulnificus infection on hematocrit values in mice. Solid lines, hematocrit values for mice injected i.p. with 2 x 10⁷ CFU of V. vulnificus; dashed lines, control mouse injected i.p. with saline; x, time of death.
TABLE 1. Nonviable materials derived from V. vulnificus which did not produce edema or lethality when injected into mice

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<th>Source of material</th>
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<th>Source of material</th>
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<tr>
<td>Culture supernatant</td>
<td>Filtration (various filters)</td>
<td>Killed cells</td>
<td>Heat (60°C for 30 min or 121°C for 15 min)</td>
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<td></td>
<td>Ultrafiltration</td>
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<td>Ultrafiltration followed by gel filtration</td>
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<td>Nutritional manipulations</td>
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<td>Disrupted cells</td>
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<td>Incubation anaerobically or in air + 5% CO₂</td>
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* Cultures were incubated in brain heart infusion or modified salt water yeast extract broth for 6 or 18 h at 35°C or at room temperature with or without shaking. Cultures were centrifuged at 10,000 × g for 20 min, and supernatants were sterilized by filtration and tested immediately in two to five mice. Preparations were injected s.c. (0.1 ml) or i.p. (0.5 ml). Some were also tested intravenously (0.1 ml). The Evans blue method was used with most s.c. injections.

* Supernatants were sterilized by filtration through 0.22-μm Millipore cellulose acetate filters. All brain heart infusion broth supernatants were also tested after filtration through polycarbonate (Nucleopore Corp., Pleasanton, Calif.) or Teflon (Tuffryn) filters (pore size, 0.45 μm).

* The filtrates described in footnote b were concentrated 10-fold by using a PM-10 membrane (Amicon Corp., Lexington, Mass.).

* A filtrate from a brain heart infusion broth-grown culture was concentrated as described in footnote c and then subjected to Sephadex G-100 chromatography as described by Sandefur and Peterson (16).

* Cells were cultured in modified salt water yeast extract or brain heart infusion broth supplemented (in separate experiments) with the following: 2.5% NaCl; 1% agar; 4% gelatin; 5% sheep erythrocytes; 0.35% pooled human serum; 0.2% collagen, chondroitin sulfate, or hyaluronic acid (Sigma); fresh mouse tendon, skin, or intestine; 0.9% rat liver homogenate; or 1% yeast extract. Cells also were cultured in casein hydrolysate salts medium containing varying concentrations of glucose and NaCl, as follows: glucose, 0 or 1%; NaCl, 0.1, 1.0, or 3.0%. These cultures were incubated at 35°C for 5 or 18 h, sterilized by cellulose acetate membrane filtration (Millipore), and then tested before and after 25-fold concentration (Amicon PM-10 membrane).

* The pH was adjusted at 24 h from 5.8 to 8.0 or was maintained between 7.2 and 7.9 during growth by periodic addition of dilute NaOH.

* Cells grown in 1.5 liters of brain heart infusion broth supplemented with an additional 2.5% NaCl (shaken overnight at 35°C) were harvested by centrifugation and then suspended in 100 ml of 0.12 M tris(hydroxymethyl)aminomethane (pH 8.5). This suspension was incubated at 35°C for 2 h and centrifuged, and the supernatant was precipitated with (NH₄)₂SO₄ (90% saturation) overnight at 4°C. The precipitate was dissolved in 5 ml of phosphate-buffered saline (pH 7.4) and dialyzed overnight against four changes of 0.1 M sodium phosphate buffer (pH 7.0).

* As described in footnote g (from a culture grown in brain heart infusion broth supplemented with an additional 2.5% NaCl without pH release).

* Sterility testing in each case was carried out by plating onto brain heart infusion agar supplemented with an additional 2.5% NaCl. Viable cells which were from the same suspension and were treated identically except for the inactivation step were used as controls in each experiment.

* Groups of 10 mice were injected s.c., i.p., or intravenously with 2 × 10⁶ heat-killed (60°C) cells in 0.1 ml of saline. The Evans blue method was used with s.c. injections. Inocula of 10⁸ autoclaved (121°C) cells were also tested by s.c. and i.p. injection.

* Westinghouse Sterilamp 59 cm above an open plastic petri dish containing 5.5 ml of a saline suspension of Vibrio cells. The exposure time was 5 min, and the inoculum was 10⁸ to 10⁹ killed cells in 0.1 ml of saline injected s.c., i.p., or intravenously into groups of 10 mice. Hematocrit values were monitored for 24 h and did not change.

* Cells were washed in saline and then suspended in 1% aqueous phenol. After incubation at room temperature for 2 h, the cells were harvested again by centrifugation, washed twice in 0.85% saline, and suspended in saline; 10⁸ to 10⁹ killed cells in 0.1 ml were injected s.c., i.p., or intravenously into 10 mice.

* A saline suspension containing 10⁹ CFU/ml was subjected to intermittent sonic oscillation in an ice bath (Branson Sonifier at 150 W and a frequency of 20 kHz or Kontes Sonifier at 10.54 W and 23.5 kHz). The resulting suspension, which contained <10⁸ viable cells per ml, was injected immediately (0.1 ml s.c. or i.p.; five mice in each experiment).
duced at maximal rates in medium containing 0.5% glucose (9). However, these and other variations in media and incubation conditions (Table 1) did not result in demonstrable toxin production by *V. vulnificus*.

The pH of brain heart infusion broth cultures of *V. vulnificus* after 24 h of incubation was 5.8. Like cholera toxin, the heat-labile enterotoxin of *E. coli* remains cell associated below pH 7.0 and is released when the medium pH is readjusted to 8.0 (9). However, attempts at "pH release" did not render *V. vulnificus* filtrates toxic (Table 1).

Sandefur and Peterson (16) described a skin permeability factor produced by *Salmonella typhimurium* which was detectable only after elution from a Sephadex G-100 column or, to a lesser extent, after concentration by ultrafiltration. These techniques were also tested in our system but had no effect (Table 1).

The failure of cells killed by a variety of methods and of crude cell extracts prepared by sonic disruption to induce vascular permeability implied that the effect was probably not attributable to endotoxin or other cell-associated products per se. The presence of an inducible cell-associated enzyme, as described by Drzeniek et al. (7) for *Pasteurella multocida*, is possible.

**Collection of edema fluid.** Because no vascular permeability factor was evident in culture filtrates or in cells grown in vitro and killed by a variety of methods, we investigated the possibility that a toxin might be elaborated only during in vivo growth. The edema which formed after s.c. injection of *V. vulnificus* consisted of a gelatinous mass beneath the skin, from which only small amounts of fluid could be collected by prodding with a pipette after the mouse was sacrificed and the skin was dissected away. To obtain sufficient edema fluid for this study, we used helical chambers of surgical steel wire implanted s.c. under ether anesthesia by the method of Arko (1). Such chambers were inserted by twisting through a single needle hole, produced minimal trauma, and provided a space for s.c. fluid accumulation. Mice were used for experiments 3 and 14 days after insertion of chambers. When viable cells of *V. vulnificus* were injected percutaneously into these chambers, visible edema formed in the surrounding tissues, and fluid accumulated inside the chambers. Such infections were fatal and had time courses similar to the time course after direct s.c. injection. The amounts of fluid obtainable from infected chambers by using a syringe and needle 4 to 5 h after injection of 10^8 CFU per mouse were eightfold greater than the amounts of fluid obtained from control chambers injected with saline (mean, 0.54 ml from 15 infected mice; mean, 0.07 ml from 9 controls). As observed by phase-contrast microscopy, fluid from infected mice contained large numbers of motile *Vibrio* cells. Viable counts of 3 × 10^5 cells per ml were obtained in the chamber fluids withdrawn at death from mice injected with 10^2 cells. Pooled chamber fluid from infected mice was sterilized by filtration and did not produce edema or skin bluing when it was injected s.c. (0.1 ml) into mice, nor were any symptoms observed after i.p. injection of 0.5 ml.

**Immunization studies.** It was possible that a toxin might be produced in vivo in amounts too small to produce visible effects after passive transfer. We investigated the possibility that such a product might nevertheless immunize recipient mice against subsequent challenge. At 3- to 4-day intervals, groups of 10 mice received four i.p. injections of 0.2 ml of chamber fluid obtained from infected or uninfected mice, or of 10^6 heat-killed (100°C, 10 min) whole cells of *V. vulnificus*. At 2 weeks after the last injection, these mice were challenged by i.p. injection of 5 × 10^6 CFU of viable *V. vulnificus* cells per mouse. All of the mice which had been immunized with heat-killed cells survived, whereas all mice immunized with normal or infected filtered chamber fluid died. When an s.c. challenge dose of 10^8 CFU/mouse was administered subsequently to the mice immunized with heat-killed cells, local skin bluing and mild edema that was much less severe than in lethal infections were demonstrable by the Evans blue method.

**Antibiotic treatment.** Tetracycline (50 μg) injected s.c. at the site of *Vibrio* injection prevented death if it was administered up to 2 h after s.c. injection of 10^6 CFU of *V. vulnificus* per mouse. When tetracycline was administered at 0, 1, 2, and 3 h after s.c. *Vibrio* injection, seven of seven, seven of eight, five of seven, and none of six mice survived for 18 h, respectively; none of seven untreated control mice survived. Mice given tetracycline at the time of *Vibrio* injection developed only mild edema and minimal skin bluing by 5 h. The degree of local edema observed in treated animals increased in proportion to the delay in treatment.

Thus, sublethal local edema was produced by virulent *V. vulnificus* under three circumstances. If the inoculum size was small (<10^6 cells) (12), if the mice were immunized previously with heat-killed cells, or if an effective antimicrobial agent was administered within 2 h after challenge, mild local edema and visible bluing resulted. However, the extent of edema was much less than in lethal infections, the mice survived, and the lesions later usually became
necrotic. As reported by Poole and Oliver (12) and confirmed here, mice receiving a lethal inoculum of *V. vulnificus* were uniformly bacteremic, with about $10^4$ to $10^5$ CFU/ml recoverable from heart blood at death. Thus, the virulence mechanisms of this organism apparently include both the ability to produce increased vascular permeability, whatever the mechanism, and the capacity for systemic invasion.

**Containment chamber.** To determine whether local skin bluing, edema, or systemic pathology could be produced in the absence of dissemination of *Vibrio* cells, these cells were contained in chambers constructed from cellulose acetate filters (pore diameter, 0.22 μm; Diffusion Chambers; Millipore Corp., Bedford, Mass.). In the first experiment, chambers containing $10^6$ CFU of *V. vulnificus* in 0.1 ml were implanted s.c. on the backs of 10 mice under ether anesthesia. Five mice received sham chambers containing only saline. The incisions were closed with wound clips. Successful implantation without contamination was achieved in five of the experimental mice; these animals showed no signs of illness and survived with chambers in place for 6 weeks. Evans blue injected i.p. shortly after implantation did not produce skin bluing over the chambers, except along the incision itself in both test and control animals. Local edema was not observed. Chamber contents aspirated from infected mice on day 5 after implantation and injected i.p. into a normal mouse resulted in death; *V. vulnificus* was recovered from the heart blood, indicating that *Vibrio* cells had remained viable and virulent within the chambers. No effect was observed in a control mouse injected with the contents aspirated from sham chambers. Fluid aspirated from chambers 2 weeks after implantation contained approximately $10^7$ CFU of *V. vulnificus* per ml (total chamber volume, approximately 0.1 ml). The *Vibrio* cells appeared primarily as spherical forms when the fluid was examined microscopically. Mice which retained the chambers for 6 weeks were challenged by i.p. injection of $10^8$ CFU of viable in vitro-grown *V. vulnificus* cells per mouse; these mice were not immune to this challenge dose and died as rapidly as sham controls.

This experiment was repeated by implantation into six mice of chambers containing $3.5 \times 10^7$ CFU to test the possibility that active growth might be required for edema production. Again, local bluing was not demonstrated by the Evans blue method. However, growth within these chambers was not demonstrated, as the small amount of fluid subsequently aspirated from them contained fewer than $10^6$ CFU/ml.

The fact that the *Vibrio* cells within these chambers remained viable for weeks without producing visible skin bluing or palpable edema suggests that actual contact of *Vibrio* cells with host tissue may be required to produce these effects; whether a permeability factor is elaborated by the *Vibrio* or by the host is not known.

As Carpenter (5) has pointed out, the rapidly progressive invasion associated with *V. vulnificus* infections in both of its recognized clinical forms is unique among *Vibrio* species, and the pathogenic mechanisms remain to be elucidated.

The mouse model studied here is similar in several respects to the infection described in humans. Rapid progression in s.c. tissue with associated edema and subsequent necrosis are characteristic of both systems. Blake et al. (4) pointed out that death in human *V. vulnificus* infections "appeared to be caused by intractable shock secondary to gram-negative sepsis;" in some patients, thrombocytopenia and the presence of fibrin split products were reported, suggesting disseminated intravascular coagulation.

The hemocentration and apparent loss of intravascular fluid observed in the mouse model may be interpreted as "shock." Mice dying of *V. vulnificus* infections appear to die from inadequate tissue perfusion; they appear cyanotic, and eventually breathing ceases, although the heart continues to contract for some time.

Our results with small inocula, immunized mice, and antibiotic therapy demonstrate that under some conditions virulent *V. vulnificus* can produce mild edema without irreversible progression to lethality. Whether the difference is solely in the extent of intravascular fluid loss or whether systemic invasion or other mechanisms are also required for lethality is not yet clear.

One strain of *V. vulnificus*, CDC-A1402 (a corneal ulcer isolate), was reported by Poole and Oliver to be avirulent in mice. We have observed that this strain is capable of producing mild local edema and bluing but is not lethal after s.c. injection of $10^8$ CFU/mouse. A comparison of this strain with more virulent strains with respect to possible virulence determinants would be of interest and is in progress in our laboratory.

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**LITERATURE CITED**


