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Experimental Pathogenicity and Mortality in Ligated Ileal Loop Studies of the Newly Reported Halophilic Lactose-Positive *Vibrio* sp.

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Laboratory animals were challenged subcutaneously, intraperitoneally, and intravenously with the halophilic lactose-positive *Vibrio*. Intraperitoneal inocula of 10^8 organisms proved to be rapidly lethal in mice, rats, and hamsters. The 50% lethal dose in ICR strain mice was estimated to be 8×10^5 live cells, injected intraperitoneally or subcutaneously. Subcutaneous inocula in mice resulted in severe local infections, characterized by gross edema, and for those animals surviving longer than 48 h, tissue necrosis. Intravenous, intraperitoneal, and subcutaneous injections of 10^8 cells in mice resulted in death within 3 to 6 h. These animals rapidly developed *Vibrio* bacteremia after injections. *V. parahaemolyticus*, studied for comparative purposes, produced no morbidity or mortality when injected subcutaneously. Injections of live lactose-positive *Vibrio* into ligated ileal loops in rats and rabbits consistently proved to be lethal with a high-density bacteremia resulting.

The halophilic bacterium, *Vibrio parahaemolyticus*, has gained worldwide attention since 1950 as the causative organism of numerous outbreaks of severe gastroenteritis (1, 5, 8, 21). More recently, reports have involved the halophilic vibrios in cases of extraintestinal infections (7, 9, 11, 14, 15, 17, 19, 22). In 1976, Hollis et al. (9), of the Center for Disease Control (CDC) in Atlanta, Ga., reported that the responsible organism for many of these infections was an unnamed *Vibrio* species. This organism is taxonomically closely related to *V. parahaemolyticus*, differing primarily in its ability to ferment lactose and the production of β-galactosidase. The organism was referred to as lactose-positive *Vibrio* (L+ *Vibrio*) by these investigators. Studies of phenotypic characteristics (2, 9) and deoxyribonucleic acid hybridization (13) indicated to Reichelt et al. that the organism is a separate species, and they used the name *Beneckea vulnifica* (13).

As of the 1976 report, CDC had received 38 clinical isolates of this species (9). Since that time an additional 18 isolates were received, including one from seafood and one from saltwater (D. G. Hollis, personal communication). These numbers, however, most likely misrepresent the actual occurrence of L+ *Vibrio*, as effective isolation and identification require media containing additional NaCl.

The reported cases of L+ *Vibrio* infections typically involve persons associated with marine or estuarine environments and result in the development of severe local infections characterized by edema, tissue destruction, septicemia, and, not uncommonly, death (7, 14, 17, 22). Unlike *V. parahaemolyticus*, the most likely portal of entry for L+ *Vibrio* is directly into the skin (7, 14, 17); however, a gastrointestinal entry cannot be excluded (17, 22).

The objective of this study was to monitor the pathogenesis of L+ *Vibrio* infections in a variety of laboratory animals and to select a suitable model for further studies.

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MATERIALS AND METHODS

Organisms. L+ *Vibrio* cultures were provided courtesy of R. E. Weaver and D. G. Hollis, CDC, Atlanta. Organisms were strains CDC A1402 (corneal ulcer isolate), B3547 (blood isolate), and C7184 (blood isolate). Unless otherwise stated, C7184 was the experimental L+ *Vibrio* strain used in the present studies. *V. parahaemolyticus* (ATCC 27519, a Kanagawa-positive isolate from a gastrointestinal infection) was used for comparative studies.

Media. Stock cultures were maintained as slants on a modified salt water-yeast extract agar (10), composed of 0.1% Proteose peptone (Difco), 0.1% yeast extract (Baltimore Biological Laboratory), and 1.5% agar in a three-salts solution (0.75 g of KCl, 7.0 g of $MgSO_4 \cdot 7H_2O$, and 23.4 g of NaCl in distilled water to final volume of 1 liter). Cultures were transferred to

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fresh medium at about 10-day intervals. Lactose reaction was tested in phenol red lactose broth (Baltimore Biological Laboratory) prepared in three-salts solution. Cells used for inocula were taken from modified salt water-yeast extract agar slants and grown in brain heart infusion broth (Baltimore Biological Laboratory), supplemented with an additional 2.5% NaCl. Thiosulfate citrate bile salts sucrose agar (TCBS, Baltimore Biological Laboratory) was used for selection of vibrios.

Animals. Institute of Cancer Research (ICR) strain female mice, 8 to 10 weeks old and 25 to 32 g, were obtained from Perfection Breeders, Douglassville, Pa. Hamsters, male and female, 12 to 16 weeks old, were of a laboratory colony. Rats were Sprague-Dawley-derived females, 250 to 350 g. New Zealand white rabbits, male and female, 8 to 11 weeks old, were used for ileal loop studies.

Inocula and LD_{50} estimation. Inocula were prepared from cells grown in brain heart infusion broth (with additional NaCl) for 12 h at 37°C. Cells were harvested by centrifugation, washed, and suspended in sterile 0.85% phosphate-buffered saline (pH 7.4). For 50% lethal dose (LD_{50}) studies, serial dilutions were prepared and four mice were injected with 0.1 ml of the suspension from each dilution. The number of injected organisms was determined by total viable counts on thiosulfate citrate bile salts sucrose agar. After 48 h, the mortality was tallied and the 50% end point was estimated by the Reed-Meunch method (12).

Ligated ileal loops. A modification of the method described by De and Chatterjee (6) was employed. Rats were anesthetized with ether, and two 4-cm segments of the ileum were ligated, with a 3-cm spacer loop between them. Each of the two test loops was injected, using a 26-gauge needle, with about 10^8 cells suspended in 0.1 ml of 0.85% saline. Rabbits were anesthetized with Fluothane (halothane, Ayerst Laboratories), and five test loops (8 to 10 cm) were ligated off with spacer loops (4 to 6 cm) between them. Each test loop was injected, as described above, with 0.2 ml of a 0.85% saline suspension, containing about 2×10^8 cells. Quantitative analysis of bacteremia was performed by total viable counts, using thiosulfate citrate bile salts sucrose agar and brain heart infusion broth (with additional NaCl).

RESULTS

As no work has previously been reported on the experimental pathogenesis of L+ *Vibrio* infections, various laboratory animals were challenged with the organism to select a suitable model for further studies. Table 1 presents a composite of these and subsequent investigations. Intraperitoneal (i.p.) injections of about 10^8 cells of L+ *Vibrio* proved to be fatal in mice, rats, and hamsters. Mice, identified from these studies as the most suitable model, were further challenged subcutaneously (s.c.), intravenously (i.v.), and by force feeding with L+ *Vibrio*, and for comparative purposes, with *V. parahaemolyticus*. The most striking difference between the effects of the two organisms occurred in the s.c. injections. Those of *V. parahaemolyticus* produced no observable ill effects, whereas s.c. injections of L+ *Vibrio* consistently resulted in severe local swelling and death. i.p. and i.v. injections of both species were uniformly lethal. Death after i.p. injections of L+ *Vibrio* occurred very rapidly, usually within 3 to 5 h. Death from *V. parahaemolyticus* injection typically resulted in 8 to 24 h. Neither organism produced mortality when ingested.

LD_{50} estimation. Injections of three strains of L+ *Vibrio* and one strain of *V. parahaemolyticus* were made to estimate the LD_{50} values in ICR mice (Table 2). Two of the L+ *Vibrio* strains (C7184 and B3547) yielded LD_{50} values between 2×10^5 and 2×10^6 live cells injected s.c. or i.p. Death from i.p. inocula of these L+ *Vibrio* strains occurred in 3 to 18 h, depending on dose. Although the LD_{50} value for s.c. injections was found to be slightly lower than for i.p. injections, death occurred more slowly (5 to 48 h). Injections of L+ *Vibrio* A1402 resulted in no mortality, even at doses of 10^8 cells. Again, *V. parahaemolyticus* produced no deaths when injected s.c., but yielded an LD_{50} value of 8×10^7 when injected i.p. All mice that died from injections of either organism were observed to have a high density of *Vibrio* bacteremia, estimated at greater than 10^4/ml of blood. After s.c. or i.p. injections, L+ *Vibrio* could be detected in the blood within 15 min.

Ileal loop studies. Due to the potent gastrointestinal effects of *V. cholerae* and *V. parahaemolyticus* and to the possibility of gastrointestinal entry in case histories of L+ *Vibrio* infections, the often employed ligated ileal loop technique was chosen as a model for investigations of possible gastrointestinal effects of L+ *Vibrio*. The injection of saline suspensions of live cells into ligated sections of the ileum was initially performed on rats in this study. Experimental protocol classically allows for the loops to "incubate" in vivo for 18 h, at which time the animals are sacrificed and the degree of fluid accumulation in the loops is recorded. When a high percentage of the rats died far short of 18 h, the surgical procedure came under immediate suspect. However, further study showed that all of the L+ *Vibrio*-challenged animals, and no others, died (Table 3) within 6 to 13 h. In addition, blood samples taken just before death from the L+ *Vibrio*-infected rats showed the same high density of *Vibrio* bacteremia as observed in infected mice.

Young New Zealand rabbits produced similar mortality when loops were injected with L+ *Vibrio* (Table 3). Five of the rabbits died within 5 to 14 h, similar to the time of death observed when rats were used. One rabbit injected with *V. parahaemolyticus* died after 17 h. The num-

TABLE 1. *Experimental mortality*[a]

Infecting organism	Mice				Rats	Hamsters
	i.p.	s.c.	i.v.	Ingestion	i.p.	i.p.
L+ *Vibrio* C7184[b]	62/62[c] (100)[d]	34/34 (100)	8/8 (100)	0/12 (0)	10/13 (77)	9/9 (100)
V. parahaemolyticus 27519[b]	19/24 (79)	0/24 (0)	6/6 (100)	0/12 (0)	NT[e]	NT

[a] Mice were challenged i.p., s.c., and i.v., and by forced ingestion with L+ *Vibrio* and *V. parahaemolyticus*. Rats and hamsters were injected i.p. with L+ *Vibrio* only.
[b] Inocula consisted of 0.1 ml of saline containing about 2×10^8 cells.
[c] Number of deaths out of total number of animals tested.
[d] Numbers in parentheses indicate percent mortality.
[e] NT, Not tested.

TABLE 2. *Estimated LD_{50} as determined by s.c. and i.p. injections of live cells in mice*

Injection	L+ *Vibrio*			*V. parahaemolyticus*
	C7184	B3547	A1402	27519
s.c.	2×10^5	9×10^5	—[a]	—[b]
i.p.	8×10^5	2×10^6	—[a]	8×10^7

[a] No observed mortality with an inoculum of 10^8 cells.
[b] No observed mortality with an inoculum of 10^9 cells.

TABLE 3. *Observed mortality in ligated ileal loop studies*

Ileal loop inoculum	Mortality[a]	
	Rats	Rabbits
L+ *Vibrio*	12/12	5/6
V. parahaemolyticus	0/6	1/4
Saline	0/3	0/2

[a] Number of animals dead before 18 h out of the total number tested.

TABLE 4. *L+ Vibrio concentration in blood of rabbits receiving ileal loop injections*

Rabbit	Time to death[a] (h)	No. of vibrios/ml of blood[b]
1	9	3×10^6
2	13	6×10^4
3	—[c]	5×10^3

[a] Amount of time elapsed after five ileal loops were injected with 0.2 ml of saline containing 2×10^8 L+ *Vibrio* cells.
[b] Assays were made within 15 min of death.
[c] Animal was sacrificed at 18 h.

ber of vibrios in the blood of three of the rabbits whose ileal loops received L+ *Vibrio* injections was quantified by total viable counts (Table 4). *Vibrio* bacteremia varied from 5×10^3 to over 10^6 cells per ml of blood within 15 min of death.

DISCUSSION

The results of this study provide the first experimental data on the pathogenesis of the L+ *Vibrio* sp. The data may be particularly significant and relevant, as they correspond well with the edema, invasiveness, bacteremia, and death reported in case histories (7, 9, 14, 17, 22). Such an appropriate in vivo experimental model may be a valuable tool in further clinical studies of this organism.

The LD_{50} values indicate that the L+ *Vibrio* is somewhat more virulent in mice than is *V. parahaemolyticus*. The one relatively avirulent strain (A1402) or L+ *Vibrio* may prove to be quite valuable in pinpointing pathogenic factors and mechanisms. The estimated LD_{50} for *V. parahaemolyticus*. The one relatively avirulent strain (A1402) of L+ *Vibrio* may prove to be quite valuable in pinpointing pathogenic factors and mechanisms. The estimated LD_{50} for *V. parahaemolyticus* 27519 is slightly higher than size discrepancy between BALB/c and ICR mice, the latter being considerably larger.

Lethal effects resulting from s.c. inoculations of L+ *Vibrio*, but not *V. parahaemolyticus* (Tables 1 and 2), strongly support this organism's unique involvement in serious extraintestinal infections. Even *V. cholerae*, when injected s.c., is not lethal (20). The edematous response has subsequently been investigated in this laboratory (submitted for publication) and appears to result from massive vascular leakage of plasma proteins. In addition, the rapid death from i.p. and s.c. injections of L+ *Vibrio* apparently stems from the effects of gross hemoconcentration and hypothesion, relating to a generalized fluid loss (submitted for publication).

The lack of mortality when mice were fed L+ *Vibrio* and *V. parahaemolyticus* was to be expected. *V. cholerae* produces no ill effects when fed to adult rodents, unless the stomach is previously alkalinized (20). The sensitivity of L+ *Vibrio* to low pH values is reflected in the inability to maintain cultures on such media as

brain heart infusion broth, which contain glucose that is readily fermented to acidic end products.

The results of the ileal loop studies must be interpreted cautiously. The ileal loops in rats and rabbits showed the same moderate fluid accumulation, with fluid appearing in blank spacer loops as well as in test loops. Although some L+ *Vibrio* test loops produced an accumulated fluid (milliliter) to loop length (centimeter) ratio of about 1, premature death of tested animals prohibit a conclusive statement concerning the ability of L+ *Vibrio* to elicit a positive fluid response in ligated ileal loops. Although Kanagawa-positive strains of *V. parahaemolyticus* have previously been reported to yield positive ileal loop results (3, 16, 18), none of the test loops from four rabbits showed any accumulation. It is significant, however, that all 12 rats and 5 of 6 rabbits whose loops received L+ *Vibrio* injections died before the prescribed 18-h incubation period. No such lethality was observed in animals that received *V. parahaemolyticus* injections (Table 3).

B. K. Boutin, S. F. Townsend, P. V. Scarpino, and R. M. Twedt (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B24, p. 19) have recently reported that *V. parahaemolyticus* can be isolated from blood after rabbit ileal loops are injected with Kanagawa-positive strains. However, they provided no quantitative data on the bacteremia, nor was any mortality reported, as with L+ *Vibrio* in our study. A rapidly occurring bacteremia was observed in three of the rabbits after ileal-loop injection with L+ *Vibrio*, but until histological studies explore the development of this bacteremia, it would be presumptuous to propose an active invasiveness by these organisms. The relative virulence reported in this study and increasing number of reported L+ *Vibrio* infections dramatically warrant further investigations into the ecology and pathogenicity of this organism.

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