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In Vivo Resuscitation, and Virulence towards Mice, of Viable but Nonculturable Cells of *Vibrio vulnificus*

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***Vibrio vulnificus* is an estuarine bacterium responsible for 95% of all seafood-related deaths in the United States. The bacterium occurs naturally in molluscan shellfish, and ingestion of raw oysters is typically the source of human infection. *V. vulnificus* is also known to enter a viable but nonculturable (VBNC) state, wherein the cells are no longer culturable on routine plating media but can be shown to remain viable. Whether or not this human pathogen remains virulent when entering the VBNC state has not been definitively demonstrated. In this study, the VBNC state was induced through a temperature downshift to 5°C, with cells becoming nonculturable (<0.1 CFU/ml) within 7 days. As they became nonculturable, virulence was determined by employing an iron overload mouse model. At the point of nonculturability (7 days), injections of the diluted microcosm population resulted in death when <0.04 CFU was inoculated, although >10^5 cells in the VBNC state were present in the inoculum. Culturable cells of *V. vulnificus*, with identification confirmed through PCR, were recovered from the blood and peritoneal cavities of mice which had died from injections of cells present in the VBNC state for at least 3 days. Thus, our data suggest that cells of *V. vulnificus* remain virulent, at least for some time, when present in the VBNC state and are capable of causing fatal infections following in vivo resuscitation. Our studies also indicate, however, that virulence decreases significantly as cells enter the VBNC state, which may account, at least to some extent, for the decrease in infections caused by this bacterium during winter months.**

Vibrio vulnificus is an estuarine bacterium that is known to be a significant human pathogen. Primarily affecting persons with serum iron overload resulting from such syndromes as chronic hepatitis and alcoholic cirrhosis, infection with this bacterium typically results in fatality rates of ca. 60%. Infection of this risk group generally follows from ingestion of raw or undercooked shellfish, especially oysters. Indeed, this single bacterium is responsible for 95% of all reported seafood-related deaths in this country (for a review, see reference 18).

In response to a temperature downshift to 5°C, *V. vulnificus* is known to enter into a viable but nonculturable (VBNC) state (reviewed in reference 19). In this state, the cells can be shown to be viable but are nonculturable on routine microbiological plating media. The role of the VBNC state is not clear, but it may represent a survival strategy in response to environmental stress. Cells of *V. vulnificus* show a dramatic modification of cellular lipids (13), proteins (14), and other macromolecules (19) on entry into this state. Whether or not these cells retain virulence, however, has not been conclusively demonstrated. Studies with *V. cholerae* (3, 4), *Campylobacter jejuni* (25), and *Legionella pneumophila* (10) have suggested that these bacteria are capable of retaining virulence when in the VBNC state. The present study was designed to investigate the ability of *V. vulnificus* to initiate infection as it enters the VBNC state. Mice were injected at various times with cells before and after they became nonculturable, and 50% lethal doses (LD_{50}s) were calculated at each time interval. The ability of cells in the VBNC state to resuscitate to the actively metabolizing state in the animal host was also examined.

MATERIALS AND METHODS

Bacteria and microcosms. *V. vulnificus* CVD713 (opaque) and C7184 (opaque) were employed in our studies. Strain CVD713 contains transposon Tn*phoA*, which imparts both kanamycin resistance and the ability to produce alkaline phosphatase. Cells of this strain produce brilliant blue colonies on a medium (Tn agar) containing kanamycin and the colorimetric alkaline phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate, allowing both rapid isolation and identification of this strain (5, 16). Following overnight growth in heart infusion (HI) broth at 37°C, cells were grown in HI broth to the logarithmic phase (optical density at 610 nm of 0.22 to 0.25). From this culture, 1 ml was transferred directly into 99 ml of sterile phosphate-buffered saline in the study with strain CVD713 or to artificial seawater (ASW; reference 31) in the study employing strain C7184. This protocol resulted in microcosms with 100 ml of a ca. 10^5 to 10^6 final cell concentration. ASW was employed in the second study because ASW better approximates the natural seawater environment of *V. vulnificus* and such microcosms are the type we have typically employed in our studies on the VBNC state of this bacterium. After time zero enumerations and mouse injections, microcosms were placed at 5°C to induce the VBNC state.

Enumeration assays. Culturability of the cells in the microcosm was determined by spread plates on HI agar. When the culturable cell population was less than 10 CFU/ml, a 10-ml aliquot of the microcosm was filtered and the filter was placed on HI agar. This allowed the culturable population to be determined at a level of <0.1 CFU/ml. Total cell counts were determined by both acridine orange direct counting (AODC; reference 22) and DAPI (4′,6-diamidino-2-phenylindole) staining (9), and cell viability was determined by both the direct viable count (DVC) method of Kogure et al. (11) and the 5-cyano-2,3-ditolyl tetrazolium chloride reduction method described by Rodriguez et al. (24). As our preliminary studies suggested that SO_4 may inhibit 5-cyano-2,3-ditolyl tetrazolium chloride reduction, our modification consisted of washing cells in SO_4-free ASW and assaying with this same solution. Further, as a nutrient source, HI broth powder (BBL) was added to a final concentration of 80 μg/ml.

Animals and LD_{50} studies. CD-1 mice, 5 to 9 weeks old, were employed in all lethality studies. Immediately prior to challenge with *V. vulnificus*, animals were iron overloaded through intraperitoneal injection of 0.2 ml of freshly prepared ferric ammonium citrate (32). Dilutions of the cells were made to 10^{-5} in phosphate-buffered saline, and then 0.5 ml of the diluted cells was injected into each of three mice per dilution. Such a protocol resulted in injection of 6.5×10^4 to $<1 \times 10^{-6}$ cells on the basis of plate counts and approximately 7×10^5 cells on the basis of total counts, depending on the age of the microcosm. Animals were monitored for 48 h, and the numbers which were alive and dead were then recorded. LD_{50}s were calculated as described by Reed and Muench (23). Two such studies were conducted.

On several occasions when mice died as a result of injections of PBS containing only VBNC cells, the peritoneal cavity and opened heart were swabbed and the material obtained was plated onto Tn agar, the CVD713-specific medium. The PCR was employed to verify that the resultant colonies were *V. vulnificus* (1, 2).

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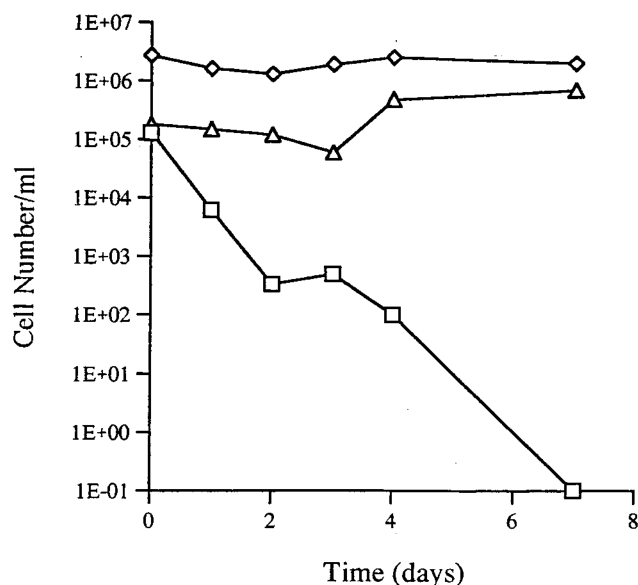

FIG. 1. Entry of *V. vulnificus* into the VBNC state in ASW at 5°C. Symbols: □, plate counts on HI agar; ◇, total direct cell counts by DAPI staining; △, DVC counts by the 5-cyano-2,3-ditolyl tetrazolium chloride reduction method. These data correspond to Table 1, where LD_{50}s of these cells for mice as the cells enter the VBNC state are shown. See Materials and Methods for details of assays.

RESULTS

Figure 1 shows the entry of *V. vulnificus* into the VBNC state induced by a temperature downshift from 24 to 5°C. While plate counts rapidly decreased to levels below detection (<0.1 CFU/ml at day 7), total direct counts remained constant at ca. 2×10^6 cells/ml. Cell viability, as evidenced by the 5-cyano-2,3-ditolyl reduction method, fluctuated between 2×10^4 and 7×10^5 cells/ml.

Because the plate count data declined continually as cells entered a VBNC state, it was difficult to calculate estimated LD_{50}s on the basis of such data. For this reason, Table 1 shows LD_{50}s estimated on the basis of plate count, total direct count, and DVC data. At the initiation of the study, an LD_{50} (based on plate counts) of 3.6 cells was obtained. Such a value is typical for log-phase cells of virulent strains of *V. vulnificus* when injected into iron-overloaded mice (27). Also evident, however, is that when plate counts were used as the basis for calculating LD_{50}s, values of less than one cell were obtained after 8 h of cell incubation at 5°C. In contrast, LD_{50}s were typically $<10^2$ when calculated on the basis of total direct counts and <10 when based on direct viable counts, until the day 4 of incubation at 5°C (Table 1). In our preliminary study, LD_{50} values based on viable counts also indicated values of generally $<10^2$ cells for the first 3 days of refrigeration (Table 2). In contrast, LD_{50}s based on plate counts suggested that lethality was occurring in mice injected with as few as 0.03 culturable cell.

On several occasions when mice died (typically within 24 h) as a result of injections of dilutions of the microcosm containing only viable but nonculturable cells, culturable cells of *V. vulnificus* CVD713 could be isolated from the peritoneal cavity and heart. Confirmation of the identity of these isolates as *V. vulnificus* was made by PCR amplification of whole cell lysates (2) of the colonies that developed.

In another study, employing a different strain of *V. vulnificus* (C7184 opaque), a zero time LD_{50} of 48 CFU was observed, whereas injection of VBNC cells (<0.1 CFU/ml after 6 days at 5°C) resulted in an LD_{50}, based on plate counts, of $<5 \times 10^{-4}$. An LD_{50} based on total direct cell counts was calculated as 5.5×10^3. Such values are typical of the data shown in Tables 1 and 2 for LD_{50}s of nonculturable cells.

DISCUSSION

A number of bacteria, including many pathogens, have been reported to be capable of entering a VBNC state (19). As with *V. vulnificus*, the natural inducer of this state in many of these bacteria appears to be low temperature. We have previously reported (1, 13, 17, 20, 21, 31) that *V. vulnificus* enters the VBNC state when incubated in ASW microcosms in the laboratory at 5°C. Evidence for and against the ability of such cells to exhibit in vitro resuscitation following a simple temperature upshift has been presented (17, 29). More recently, however, we have provided evidence that *V. vulnificus* undergoes entry into, and resuscitation from, the VBNC state in natural estuarine environments (7). The results provided in the present study suggest that resuscitation of *V. vulnificus* cells can also occur in mice and lead to fatal infection. This conclusion is based on the fact that in all of our experiments, microcosm inocula, dilutions of which totally precluded the possibility that any culturable cells were present, generally resulted in mouse death (Table 1, plate counts). Only when total direct count or DVC count data are considered and LD_{50}s are calculated on the basis of these assays do the injected cell numbers parallel those described for *V. vulnificus* in iron-overloaded mice (15, 33). This model reflects epidemiological (reviewed in reference 18) and experimental (6, 15, 26, 32, 34) data which strongly

TABLE 1. LD_{50}s calculated for *V. vulnificus* cells as they enter the VBNC state

Time of counting[a]	LD_{50} calculated from:						
	Plate counts	Total direct counts[a]			Direct viable counts[a]		
		DAPI	AODC	$\bar{x}$[b]	CTC[c]	DVC	$\bar{x}$[b]
0 h	3.6	7.5×10^1	3.9×10^1	5.7×10^1	4.3	0.6	2.5
8 h	1.3	2.4×10^2	2.3×10^2	2.4×10^2	3.3	3.0	3.2
24 h	0.2	5.4×10^1	4.7×10^1	5.1×10^1	5.1	1.1	3.1
2 days	0.02	6.5×10^1	7.0×10^1	6.8×10^1	6.0	—[d]	6.0
4 days	0.5	1.3×10^4	5.0×10^3	8.1×10^3	2.4×10^3	1.8×10^2	6.6×10^2
7 days	0.039	7.4×10^5	—	7.4×10^5	2.7×10^5	—	2.7×10^5

[a] See Materials and Methods for assay descriptions.
[b] Geometric mean of the two assays.
[c] CTC, 5-cyano-2,3-ditolyl tetrazolium chloride.
[d] —, no data.

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TABLE 2. Virulence of *V. vulnificus* C7184 during entry into VBNC state

Time of counting	LD_{50} calculated from:				
	Plate counts	Total direct counts[a]			DVC[b]
		DAPI	AODC	$\bar{x}$[c]	
0 h	9.1×10^1	8.9×10^3	1.7×10^3	5.3×10^3	2.0×10^1
4 h	5.0	5.0×10^2	1.3×10^3	3.4×10^3	1.8×10^1
8 h	3.8	2.1×10^3	5.0×10^3	3.6×10^3	4.3×10^1
24 h	0.6	2.6×10^4	1.4×10^4	2.0×10^4	2.6×10^2
2 days	0.03	—[d]	1.3×10^4	1.3×10^4	7.5×10^1
3 days	0.15	1.6×10^4	6.0×10^4	3.8×10^4	2.0×10^2

[a] See Materials and Methods for assay descriptions.
[b] DVC counts were determined by the method of Kogure et al. (1).
[c] Geometric mean of the two assays.
[d] —, no data.

indicate a correlation between infection with *V. vulnificus* and the presence of one of several diseases which result in serum iron overload. Further, the isolation of culturable cells of this species from within dead mice injected with only cells in the VBNC state strongly indicates that such cells are able to resuscitate in the mammalian host and cause death.

V. vulnificus possesses an acidic polysaccharide capsule which has been shown to be antiphagocytic (12, 28) and appears to be essential to the virulence of this organism (26). We had previously reported (13) that this capsule is retained during entry into the VBNC state by this bacterium, and preliminary evidence (data not shown) employing direct capsule staining with a polyclonal antibody appears to confirm this. Such capsule retention is likely essential to successful resuscitation in, and infection of, susceptible animals.

Epidemiological evidence indicates that most cases of *V. vulnificus* infection occur in the summer months, when water temperatures are highest (18). Nevertheless, cases have been reported when water temperatures are low. For example, 28% of the 138 cases reported in five Gulf Coast states between 1988 and 1993 (30) and 14% of the 79 cases reported in Florida between 1981 and 1993 (8) occurred between October and April. During the winter months, water temperatures are frequently <14°C in Gulf Coast waters, and at these temperatures *V. vulnificus* is not detected in either the water column or oyster meats (23a). In this respect, it is interesting that both total direct count- and DVC count-based LD_{50}s observed in the present studies changed little during the first 2 to 3 days of incubation at 5°C and then increased dramatically. These results suggest that while *V. vulnificus* maintains its ability to initiate potentially fatal infections during entry into the VBNC state, its virulence decreases over time. The data presented here thus offer a possible explanation of why we did not observe mouse lethality in a previous study (13) which employed cells injected after 3 weeks of incubation at 5°C. The decrease in virulence observed over time may account for the greatly reduced incidence of infections caused by this bacterium reported during winter months.

We believe that our results are the first to employ extensively diluted cell populations to rule out the presence of culturable cells in the mouse inocula employed. The use of such dilutions, coupled with characterization of the injected populations by plate counts, total direct counts, and DVC counts, indicates that only cells of *V. vulnificus* in the VBNC state were injected. The resuscitation and death that followed these injections indicate that a public health concern may indeed be present when pathogens exist in the VBNC state.

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