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Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine

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***Vibrio cholerae* O1 can enter a state in which they remain viable but are non-culturable. Presumably, such bacteria can be pathogenic if they retain the capacity to proliferate in the human intestine following ingestion. Two groups of volunteers were given inocula containing viable but non-culturable *V. cholerae* O1 of the attenuated vaccine strain CVD 101 (viable CVD 101 organisms readily colonize the human intestine). Volunteers in one of the two groups excreted viable CVD 101, demonstrating that, in the environment of the human intestine, previously non-culturable vibrios can regain the capacity to multiply. These observations support the proposition that viable but non-culturable bacterial enteropathogens may pose a potential threat to health.**

Key words: Cholera, viable but non-culturable, *Vibrio cholerae*.

Results of microcosm and field studies show that many Gram-negative bacterial pathogens, including *Vibrio cholerae*, can enter a state in which they remain viable but cannot be cultured by any technique currently available (Xu *et al.* 1983; Roszak *et al.* 1984; Rollins & Colwell 1986; Byrd *et al.* 1991). Attempts have been made to culture these organisms, without much success. Non-culturable cells of *V. cholerae* can be detected in significantly greater numbers than culturable cells in natural water samples when direct detection methods, such as those involving fluorescent-labelled monoclonal antibodies, are used (Brayton *et al.* 1986; Huq *et al.* 1990). The non-culturable state into which *V. cholerae* enters and the capacity to persist in the aquatic environment are important features in this vibrio's ecology. It has been suggested that cells of *V. cholerae* attached to plankton enter the non-culturable stage as an environmental survival strategy (Colwell *et al.* 1985; Roszak & Colwell 1987; Colwell & Huq 1994).

The maintenance of virulence in non-culturable organisms has not been widely studied. For example, Beumer *et al.* (1992) were unable to demonstrate typical symptoms of campylobacteriosis in laboratory animals and human volunteers, and concluded that the reversion of coccoid cells to normal viable cells may require specific conditions, if it occurs at all. That cells of *V. cholerae* O1 remain potentially pathogenic while in the non-culturable state (Colwell *et al.* 1985, 1990) has tremendous public health interest, particularly in areas endemic for cholera. As far as we are aware, all previous attempts to demonstrate retention of virulence of *V. cholerae* have involved animals other than man. The current study was undertaken to demonstrate the capacity of non-culturable cells of attenuated *V. cholerae* O1 to colonize while passing through the intestinal tracts of healthy, human volunteers.

Materials and Methods

Microcosm for Challenge Inoculum

A series of microcosms was assembled in the laboratory to obtain

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several 100–ml volumes of non-culturable cells of an attenuated strain of *V. cholerae*, CVD 101, which had previously been shown to colonize the human intestine readily (Levine *et al.* 1988). This strain was derived from wild-type strain 395 (of classical biotype and Ogawa serotype) by deletion of 94% of *ctxA*, the gene encoding the A (ADP-ribosylating) subunit of cholera toxin (Kaper *et al.* 1984). Although CVD 101 is clearly attenuated compared with its wild-type parent, and unable to cause cholera gravis, in extensive clinical trials, this vaccine strain nevertheless elicited mild diarrhoeal adverse reactions in approximately 50% of volunteers who each ingested 10^3 to 10^8 c.f.u. in $NaHCO_3$ buffer (Levine *et al.* 1988).

Cells of *V. cholerae* O1 strain CVD 101 were grown in alkaline/peptone water at 37°C. Bacterial culture from mid-log phase was centrifuged at 3000 × *g* for 10 min and resuspended in phosphate buffered saline (PBS), pH 7.3, 8.5 g NaCl and 1.5 g Na_2HPO_4/l. Washed cells were then inoculated into 1–l volumes of PBS in each of several 4–l flasks, to give a final concentration of approx. 10^8 cells/ml, determined by the acridine orange direct count (AODC) method (Hobbie *et al.* 1977). These microcosms were then incubated at 4°C with gentle shaking, to induce the non-culturable state in the cells. Ten-ml aliquots were removed twice weekly and tested for culturability by: (1) plating on thiosulphate citrate bile-salt sucrose (TCBS) agar (Oxoid) and tryptic soy/agar (TSA) (Difco); and (2) enrichment for 6 h in double-strength alkaline peptone broth (APB) [containing 1% (w/v) each of Tryptone and NaCl, at pH 7.3], followed by streaking onto TCBS agar and TSA plates. Both the enriched broth and the plates were incubated at room temperature and at 37°C, with daily observation for growth up to 7 days, after which empty plates were deemed negative.

Bacterial Suspension given to Volunteers

Ten days after all the cells were considered to have entered into the non-culturable state, eight, 60-ml aliquots of the microcosms were removed and transferred, each to a separate flask containing 6 ml 10% (w/v) Brewer's yeast (Golden Harvest), and incubated at 37°C for 48 h to prepare the feeding inoculum. [Results of earlier experiments had shown that a suspension of Brewer's yeast (Difco) was as good an enrichment broth as the Tryptone solution used in APB.] Aliquots were again removed at 24 h and tested for growth on TCBS and TSA plates, and in double-strength APB. To confirm that all the cells given to volunteers were viable but non-culturable, the original flasks were also incubated for an additional 24 h, after addition of fresh sterile broth to the empty flasks, and tested for growth using the above media and by direct viable counting (DVC) (Kogure *et al.* 1979). A yeast-enriched broth was only given to volunteers if both the broth and the flask from which it was inoculated did not produce any colonies on the agar plates or growth in the liquid media.

Yeast-enriched broths were pooled and then distributed into flasks at approximately 100 m/flask. Aliquots were again drawn from each of these flasks and transferred to TCBS agar and TSA plates and double-strength APB to re-test for the presence of culturable cells. In addition, a 10-ml sample was removed from each flask and transferred to a sterile tube for continued incubation at 37°C for an additional 2 weeks, to ensure that no culturable cells were present in the cultures. Aliquots were also removed for AODC and DVC, to enumerate the total number and viability of the cells, respectively.

Volunteers for the Study

Volunteers were adults aged 18 to 45 years, recruited from the Baltimore community, whose good health status was ascertained by physical examination, medical history and laboratory tests (Levine *et al.* 1988). The clinical protocol was approved by the Institutional Review Board of the University of Maryland School of Medicine. Volunteers were admitted to the Research Isolation Ward maintained by the Center for Vaccine Development within the University of Maryland Hospital. Two volunteers participated in the first study, which was undertaken as follows: 150 ml $NaHCO_3$ (13.3 g/l distilled water) solution was given to each of the volunteers 1 min before he/she drank 90 ml culture containing viable but non-culturable cells of *V. cholerae*/ml. The volunteers refrained from food or drink for 90 min before and after this challenge. Presence of viable cells of *V. cholerae* was determined by collecting subsequent stools and culturing samples of them by standard clinical microbiological techniques known to be sensitive for detecting *V. cholerae* O1 in the volunteer model (Rennels *et al.* 1980).

In a second separate study, seven additional volunteers were similarly challenged, each with 100 ml suspension of viable but non-culturable cells from a second set of microcosms prepared under identical environmental conditions as before but incubated for an additional 4 weeks. These volunteers were treated as the previous two volunteers.

Results

Ten-ml aliquots each of the challenge inocula were added to 10 ml double-strength APB for enrichment and incubated for 6 h at room temperature before plating onto agar plates. Plates and broth tubes were incubated at room temperature and examined for growth of vibrios, daily for 2 weeks. In the first study, AODC showed that volunteer 1 received 1.1×10^8 *V. cholerae* cells/ml, of which 4.7×10^6 cells/ml were viable but non-culturable as determined by DVC at the time of challenge. Volunteer 2 ingested 1.5×10^8 *V. cholerae* cells/ml, with 5.6×10^6 viable cells/ml, as determined by AODC and DCV, respectively. Volunteer 1 developed mild diarrhoea and passed 751 ml watery stool 47 h post-challenge. The stool contained 2.9×10^3 culturable *V. cholerae* O1 cells/ml stool, as determined by colony count on TCBS plates. This count increased to 6×10^4 *V. cholerae* cells/ml on day 5. Although volunteer 2 did not develop diarrhoea, several yellow colonies appeared on TCBS plates streaked with stool samples recovered from him on days 4 and 5 post-challenge and these were confirmed to be of *V. cholerae* O1, by biochemical and serological tests.

In the second challenge, none of the seven volunteers apparently excreted culturable cells of *V. cholerae* as none was recovered when their stools were plated on TCBS and TSA. However, five of the seven volunteers excreted viable but non-culturable *V. cholerae* cells in their stools, as determined by substrate-responsive DVC (Kogure *et al.* 1979) employing a monoclonal antibody, raised against the A antigen of the lipopolysaccharide of *V. cholerae* O1 (Colwell *et al.* 1990). In concurrence with the bacteriological findings, none of the seven volunteers in the second study manifested diarrhoeal adverse reactions.

Discussion

We undertook studies in volunteers to determine whether the ingestion of viable but non-culturable cells of *V. cholerae* O1 would revive in the human intestine and revert to a culturable state. For ethical reasons, attenuated recombinant vaccine strain CVD 101, which does not express cholera toxin, was selected (Kaper *et al.* 1984; Levine *et al.* 1988). Although this vaccine strain is incapable of causing severe diarrhoea, previous dose-response trials in volunteers had shown that approximately 50% of individuals who ingested the vaccine strain (irrespective of dose) developed diarrhoea and that *V. cholerae* cells colonized the intestine of > 90% of the volunteers (as determined by positive stool cultures on one or more days) (Levine *et al.* 1988). Jones *et al.* (1991) were able to recover viable but non-culturable *Campylobacter jejuni* which were infectious to mice. However, Eaton *et al.* (1995) recently reported that they were unable to demonstrate colonization of coccoid *Helicobacter pylori* in gnotobiotic piglets, although bacterial adherence or colonization is known to be prerequisitive for causing diarrhoea (Moon & Runnels 1984).

In the present study, two results were observed in the two separate challenges. In the first challenge, one of two volunteers who ingested the inoculum containing viable but non-culturable vibrios subsequently excreted culturable bacteria that grew readily on TCBS, demonstrating a resuscitation in the gut. The environment of the human intestine led to a fundamental alteration in the state of the bacterial cells. Moreover, the rejuvenated bacteria interacted with the intestinal mucosa in such a way that one of the two volunteers developed mild diarrhoea, indicating that the colonization had taken place in the intestine.

In contrast, none of seven subjects in the second challenge excreted culturable *V. cholerae* O1 nor did any manifest adverse reactions to the vaccine strain. One possible explanation for the divergent results observed in the two challenges relates to the length of time that the microcosm was in the viable but non-culturable state. In the first study, the microcosm had been culture-negative for only 23 days prior to feeding to volunteers, whereas the microcosm used in the second study had been non-culturable for an additional 4 weeks. This would suggest that there may be a finite period after which viable but non-culturable *V. cholerae* O1 are less sensitive to the available triggering actions of human intestine that can re-induce a culturable state.

Viable but non-culturable *V. cholerae* O1 were present in surface (i.e. river and pond) waters in a cholera-endemic area of Bangladesh. The report of non-culturable cells of *V. cholerae* O1 producing fluid accumulation in rabbit ligated loops (Colwell *et al.* 1985) was the first demonstration of the pathogenicity of the non-culturable cells. It appeared that non-culturable cells of enteric bacterial pathogens could pose a risk to public health if such cells were present in food or drinking water. The presence of non-culturable but viable bacteria in drinking water was subsequently demonstrated by Byrd *et al.* (1991).

Results of earlier experiments (unpublished work) showed that when the cells of *V. cholerae* are grown in a large volume, incubation for a week or longer is required before they enter into the viable but non-culturable state, whereas cells grown in small volumes, e.g. 10 ml in 100 ml flasks, enter the viable but non-culturable state within a few days of incubation. However, a convincing explanation for the requirement for small volumes is yet to be determined.

The virulence of viable but non-culturable bacteria has been studied by various investigators. Although Linder & Oliver (1989) apparently failed to demonstrate virulence after viable but non-culturable cells of *V. vulnificus* were injected into mice, Oliver (1993) mentioned that a small inoculum of such cells (5×10^4) could have been a dose too low to have a lethal effect on mice. Other organisms, such as *C. jejuni*, were resuscitated to a culturable and fully virulent form by passaging the organisms through a susceptible host (Saha *et al.* 1991). In subsequent studies, Beumer *et al.* (1992) provided additional evidence for the infectability of non-culturable coccoid forms of *C. jejuni*, by measurement of ATP and DVC. However, they were unable to observe any symptoms of campylobacteriosis in laboratory animals and human volunteers challenged orally with such cells (Beumer *et al.* 1992). Medema *et al.* (1992) were also unable to demonstrate colonization of young chicken intestine by orally introducing viable but non-culturable *C. jejuni*. However, a recent study with viable but non-culturable cells of *Shigella dysenteriae* showed that such cells continued to produce shiga toxin, detected by ELISA using affinity-purified, polyclonal antibody against the β-subunit of the toxin (Rahman *et al.* 1995).

Recent studies (Ravel *et al.* 1994) have yielded transposon mutants of *V. cholerae* demonstrating an altered, viable but non-culturable response, allowing analysis of the genetic regulation of the viable but non-culturable state in this bacterium. Further studies are, however, needed to understand the actual mechanism that is involved in the conversion of culturable cells to non-culturable cells and *vice versa*.

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