Nontoxigenic *Vibrio cholerae* O1 Serotype Inaba Biotype El Tor Associated with a Cluster of Cases of Cholera in Southern India

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Thirty strains of *Vibrio cholerae* O1 belonging to the Inaba serotype El Tor biotype isolated from patients during an outbreak of cholera in the town of Warangal in southern India were found to be nontoxigenic (NT), since they did not produce cholera toxin or hybridize with DNA probes specific for cholera toxin, Zot, or Ace. The unheated and heated culture supernatants of the NT *V. cholerae* O1 evoked a rapid cell-rolling effect when introduced on confluent layers of CHO and HeLa cells which could not be inhibited by antisera against known toxins. Culture supernatants of two representative NT *V. cholerae* O1 strains caused an increase in short-circuit current in rabbit ileal tissue mounted on an Ussing chamber, and the pattern of increase in short-circuit current was consistent with the presence of a quickly acting toxin like stable toxin. None of the strains of NT *V. cholerae* O1 hybridized with a DNA probe specific for the heat-stable enterotoxin of *V. cholerae* non-O1, nor did the factor produced by NT *V. cholerae* O1 resemble the recently described heat-stable enterotoxin produced by enteroaggregative *Escherichia coli* as determined by a PCR assay. To our knowledge, this is the first report of NT *V. cholerae* O1 being associated with a cluster of cases of cholera, and it appears that a clone of NT *V. cholerae* O1 has the potential to cause localized outbreaks of cholera.

Biochemically and serologically indistinguishable strains of *Vibrio cholerae* belonging to the O1 serogroup which do not produce cholera toxin (CT) and which lack the toxin structural genes are currently termed as nontoxigenic (NT) strains. From time to time, NT *V. cholerae* O1 has been isolated from patients and from environmental sources in several countries, including Bangladesh, Guam, Brazil, Peru, Japan, England, and the United States (17). The NT strains of *V. cholerae* O1 have remained a scientific curiosity in the absence of a proper understanding of the mechanism of pathogenesis and also because of the lack of knowledge of what factors interplay in initiating acute secretory diarrhea. These strains are, however, important to bridge the hiatus in our knowledge of why recombinant attenuated candidate vaccine strains of *V. cholerae* O1 lacking all known putative toxigenic factors (21) are still able to cause residual diarrhea.

The past few years have witnessed unprecedented changes in the history of cholera. Foremost among these changes was the emergence of a new serogroup causing cholera (3) classified as *V. cholerae* O139 Bengal (20). At the time when the O139 serogroup was in the process of rapidly spreading through vast areas of India (11), a set of strains of *V. cholerae* isolated from a cholera outbreak in southern India were referred to us at the National Reference Center for Cholera in Calcutta, India. Characterization of these strains revealed them to be nontoxigenic, and to our knowledge they are the first NT strains of *V. cholerae* O1 associated with a cluster of cases of cholera. In this study, we report the extensive characterization of the strains and describe a putative factor which may be associated with the pathogenesis of these NT *V. cholerae* O1 strains.

### MATERIALS AND METHODS

**Bacterial strains.** In June 1993, we received a representative set of 18 strains, in two lots, tentatively identified as *V. cholerae* from an outbreak of cholera which occurred in the city of Warangal, located in southern India in the state of Andhra Pradesh. The 18 strains, isolated between 26 April and 9 May, 1993, were from different patients and were referred to us by M. Leela Sri Ram, Department of Microbiology, Kakatiya Medical College, Warangal, India. The identity of these strains was confirmed by a variety of biochemical, physiological, and serological tests, as described previously (12). In addition, strains identified as *V. cholerae* O1 were biotyped according to the standard procedure (15).

**Production of CT.** The medium used for assessing production of CT was Casamino Acid-yeast extract (CAYE) medium supplemented with 90 μg of lincomycin (Sigma Chemical Co., St. Louis, Mo.) per ml. The test strains were cultivated at 37°C for 24 h in a rotatory shaker (Firstek Scientific, Tokyo, Japan) set at ca. 200 rpm. After centrifugation at 4,000 × g for 20 min at 4°C, the culture supernatant was used for the assay of CT by the highly sensitive bead enzyme-linked immunosorbent assay (ELISA) (14) with appropriate positive and negative controls as described previously (16).

**Tissue culture assay.** For the tissue culture assay, AKI medium (1.5% Bacto Peptone, 0.4% yeast extract, 0.5% NaCl, 0.3% filter-sterilized NaHCO3, pH 7.4) was used to grow the strains at 37°C for 24 h in a shaker. The choice of AKI medium was dictated by our previous studies which showed that AKI medium inhibited the production of the El Tor hemolysin (18). The culture supernatant obtained by centrifugation at 4°C was made cell free by using 0.22-μm pore-size disposable filter units (Sigma). The cell-free culture filtrate (CCFF) was used for the tissue culture assay. CHO and HeLa cells were grown as monolayers in Dulbecco’s Minimum Essential Medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% (vol/vol) horse serum (Gibco Laboratories, Grand Island, N.Y.). The cell lines were maintained in tissue culture flasks (25 cm²) at 37°C in a humidified 5% CO2 atmosphere. Aliquots (50 μl) of CCPF of the test strains, serially diluted in Hanks' balanced salt solution (Gibco Laboratories), were added to each well of 96-well tissue culture plates. A confluent monolayer of CHO or HeLa cells grown for 3 to 4 days was removed from the tissue culture flasks by using 0.025% trypsin-EDTA solution, and 200 μl of the cell suspension (ca. 4 × 10⁴ cells) was added to each of the 96-well plates and incubated as described above. Morphological changes in CHO or HeLa cells
were recorded at 24 h. Control wells received the unincubated culture medium as a negative control and pure CT (Sigma) as the positive control.

Inhibition of tissue culture activity with antibody. Antibodies against CT, labile toxin (LT), of enterotoxigenic Escherichia coli, El Tor hemorrhagic, verotoxin 1 (VT1), and VT2 and a monoclonal antibody against the heat-stable enterotoxin of V. cholerae non-O1 (NAG-CT) (2F) (22) were used to determine if tissue culture activity could be inhibited by the antigens. Serially diluted culture supernatant was mixed with an equal volume of an excess amount of the different antibodies separately and incubated for 2 h at 37°C. The incubated sample was then subjected to a tissue culture assay.

Hemolysis assay. Hemolytic activity of the test strains with erythrocytes from rabbits was determined as described previously (10).

Ussing chamber experiment. The test strains, along with a negative control (E. coli HB101) and a positive control (enteroaggregative E. coli [EAggEC] 17-2), were cultured in 150 ml of Luria-Bertani (LB) broth and incubated at 37°C with shaking (200 rpm). The CFCF (obtained by centrifugation at 5,000×g for 20 min and passage through a 0.22-μm-pore-size disposable filter) was used to determine if tissue culture activity could be inhibited by the antigens. Serially diluted culture supernatant was mixed with an equal volume of an excess amount of the different antibodies separately and incubated for 2 h at 37°C. The incubated sample was then subjected to a tissue culture assay.

RESULTS AND DISCUSSION

Extensive biochemical, physiological, and serological characterization of the 18 strains received from southern India revealed that 13 strains belonged to the El Tor biotype Inaba serotype of V. cholerae O1, 4 belonged to the recently described O139 serogroup, and 1 strain belonged to the non-O1, non-O139 serogroup. Using the highly sensitive CT bead ELISA, we further determined that none of the 13 strains of V. cholerae O1 or the single non-O1, non-O139 strain produced CT while all the 4 strains belonging to the O139 serogroup produced CT. All the 18 strains examined in this study produced hemolysin which was active against rabbit erythrocytes. The above findings prompted us to investigate the probable cause of cholera caused by the El Tor O1 strains which did not produce CT. A battery of DNA probes specific for several of the known toxin genes were used. None of the 13 V. cholerae O1 strains hybridized with DNA probes specific for CT, Zot, or Ace but hybridized with the DNA probe specific for El Tor hemolysin. Therefore, it was clear that the V. cholerae O1 strains did not produce CT, nor did they possess the mobile core dynamic genetic element currently described as the virulence cassette (24), and by definition they were therefore labelled as NT.

Sterile unheated and heat-treated (100°C for 10 min) CFCF of all the V. cholerae O1 strains grown in AKI medium when introduced onto confluent layers of CHO and HeLa cells evoked a rapid cell-rounding effect with complete disruption of the monolayer (Fig. 1) which could not be inhibited by antiserum against CT, LT, or VT1 and VT2 or by an anti-NAG-CT monoclonal antibody. An additional DNA probe specific for NAG-
ST was subsequently used because it was apparent that the factor evoking cell rounding was heat stable. None of the O1 strains hybridized with the above-described probe. We further examined if the heat-stable factor produced by the NT V. cholerae O1 strains resembled the recently described heat-stable toxin with the acronym EAST produced by EA4 (19). This was accomplished by using a PCR assay, from which it was clear that oligonucleotides specific for the EAST gene (19) did not produce a band of the appropriate size (98 bp) as they did with the positive-control EA4 strain, EA4.

The culture filtrates of two representative NT V. cholerae O1 strains (WO5 and WO8) were further tested on rabbit ileal mucosa mounted in Ussing chambers. Maximal change in Isc expressed in microamperes as the mean ± standard error is shown in Table 1. There was a small increase in Isc when the unoinoculated broth was added to the chamber; no additional increase in Isc was caused by the culture supernatant of E. coli HB101. Both the test strains caused an increase in Isc which was manifested relatively quickly. The pattern of change in Isc with WO5 was different from that with WO8 in that there was a very rapid increase in Isc which then gradually diminished with time. This pattern was consistent with tissues from all five rabbits used and represents the presence of a quickly acting toxin like STI.

At this point, we are not sure whether the factor of NT V. cholerae O1 eliciting cell-rounding activity and that evoking toxin activity are the same or represent two distinct entities. If these two activities are the manifestation of a single entity, then it would simplify the assay procedure to detect the toxin in the Ussing chamber experiment is difficult to perform even in a specialized laboratory. Toxins of V. cholerae O1 like the recently discovered Aae increase Isc in rabbit ileal tissue mounted in Ussing chambers by increasing the potential difference across the epithelial membrane (24). The NT V. cholerae O1 strains examined in this study were devoid of ace and ctx, which indicates that these strains produce hitherto-undefined factors with impressive Isc activity and therefore could induce diarrhea by a mechanism similar to that of classic enterotoxins. We are currently attempting to purify this toxin.

To our knowledge, this is the first report of NT V. cholerae O1 being associated with a cluster of cases of cholera. The 13 NT strains of V. cholerae O1 were isolated in the span of 2 weeks from different patients, indicating a local outbreak of cholera caused predominantly by the NT strains of V. cholerae O1. Although the existence of NT V. cholerae O1 as an entity is certain, the mechanism by which these strains induced the secretory diarrhea indistinguishable from cholera is uncertain.

Examination of several clinical and environmental strains of NT (non-CT-producing) V. cholerae O1 in a genetically defined and immunocompetent adult mouse model has revealed that certain strains (about 41%) are enteropathogenic for adult mice and may have pathogenic potential for humans through an as-yet-unknown mechanism (9). V. cholerae O1 strains that lack the toxin gene were also shown to release four times the amount of endotoxin as strains that produce the toxin (1).

The public health importance of NT V. cholerae O1 is uncertain at present, but the fact that these strains were associated with a cluster of cases of cholera-like secretory diarrhea in southern India is ominous. While precise data on the severity of the disease caused by NT V. cholerae in comparison with that caused by its toxigenic counterpart are not available, the disease caused by NT V. cholerae O1 was severe enough to require hospitalization, since all the NT strains in the study were recovered from hospitalized patients. It would be interesting to examine the vibriocidal titer of sera of patients who were infected by NT V. cholerae O1 and to examine whether the sera of these patients were also vibriocidal to heterologous toxigenic V. cholerae O1 strains. However, since the NT status of these strains was retrospectively determined, we were unable to compare the severities of the diseases or to examine the vibriocidal titer, but future studies of NT V. cholerae O1 should be aimed at this. Strains of V. cholerae O1 apparently showing similar traits, including the lack of known virulence genes, have recently been isolated from northern Brazil and designated the Amazonian variant of pathogenic V. cholerae O1 (5). Molecular epidemiological studies have shown diversity among NT V. cholerae O1 strains (2, 4) and have also shown that some of the NT strains were indistinguishable from their toxigenic counterparts (2). On the basis of the present study, it appears that there exists a clone of NT V. cholerae O1 which has the potential to cause localized outbreaks of cholera, and such strains need to be monitored carefully.

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