Response of wild-type mutants of *Vibrio cholerae* O1 possessing different combinations of virulence genes in the ligated rabbit ileal loop and in Ussing chambers: evidence for the presence of additional secretogen

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Five wild-type mutant strains of *Vibrio cholerae* serogroup O1 that lacked the CTX virulence cassette, or contained a natural deletion of a virulence gene within the CTX virulence cassette, or possessed an additional virulence gene, along with a prototype toxigenic strain representing the El Tor classical biotypes were examined by in-vivo and in-vitro methods to determine their enterotoxic potential. The ability of whole cells and culture supernates of the strains to cause fluid accumulation in the rabbit ileal loop model revealed a pattern consistent with the presence of the various virulence gene(s), with those possessing the intact CTX virulence cassette being the most secretogenic. Culture supernates of strains without the CTX virulence cassette or the strain with an incomplete cassette were also able to evoke mild to moderate fluid accumulation in the rabbit ileal loop. Of the various media used, AKI and brain heart infusion broth appeared to support the production of a hitherto unknown secretogenic factor, because culture supernates of the non-toxigenic *V. cholerae* O1 strains showed higher fluid accumulation ratios when grown in these media than in the others. To confirm that the fluid accumulation elicited by these strains in the ileal loop was due to enterotoxin activity, the effect of supernate of the strains was examined in rabbit small intestine mounted on Ussing chambers. Increases in short circuit current and tissue conductance, as compared with the medium control, were observed even with the strains that did not possess the CTX virulence cassette, confirming their ability to disrupt the function of intestinal tissue. From these studies, it was concluded that strains of *V. cholerae* O1 devoid of the CTX virulence cassette were still able to elicit a secretory response in the ileal loop and displayed enterotoxic activity in an in-vitro experimental model.

Introduction

Cholera, an acute disease characterised by copious uncontrolled purging of ‘rice-water’ stools leading to severe electrolyte depletion, dehydration, acidosis, shock and, if left untreated, to death, is caused by *Vibrio cholerae* belonging to either the O1 or O139 serogroups [1, 2]. The clinical state of cholera is principally attributed to an oligomeric protein known as cholera toxin (CT) that activates adenylate cyclase, resulting in increased levels of cyclic AMP leading to hypersecretion of salt and water [3]. A mélange of other putative toxins produced by *V. cholerae*, such as the El Tor haemolysin [4, 5], zona occludens toxin (Zot) [6] and accessory cholera toxin (Ace) [7] are believed to exacerbate the fluid secretion process and thereby contribute to the disease, but in ways not precisely discerned as yet. A dynamic 4.5-kb core region [8], termed the CTX virulence cassette [7], has now been identified in toxigenic *V. cholerae* O1 and O139 strains that, apart from carrying the CT genes (ctxAB), also carries the gene encoding a pilin-like
intestinal colonisation factor [9], the gene encoding Zot [10] and the gene encoding Ace [7]. Most recent studies show that the structural genes for CT reside on the genome of a filamentous, lysogenic phage called CTXφ [11].

The development of a safe, attenuated, live oral recombinant cholera vaccine has been plagued with the problem of reactogenicity of the vaccine strains manifested as residual diarrhoea. Various attenuated V. cholerae O1 recombinant vaccine candidates have been constructed over the past decade. These strains were clearly incapable of causing severe diarrhoea, but were still capable of inducing unacceptable levels of mild diarrhoea in human volunteer studies [12, 13].

However, it must be mentioned that one recombinant V. cholerae O1 vaccine strain (CVD103.HgR) derived from the classical Inaba strain 569B has proved to be well tolerated and immunogenic in extensive trials in different countries [14]. A recent study examined a large collection of strains of V. cholerae of diverse origins with a battery of DNA probes specific for CT, Zot, Ace and El Tor haemolysin and was able to detect strains of V. cholerae O1 with natural deletion of the ctxA gene within the CTX virulence cassette [15]. The availability of these wild-type mutants of V. cholerae O1 provided the impetus for the present study. The objective was to assess the enterotoxicity of these wild-type mutants to see whether these strains were capable of disrupting the function of intestinal tissue and thereby determine if other secretogens are involved in the pathogenesis of V. cholerae O1. Such a study could provide useful insights into the well-noted reactogenicity of vaccine strains of V. cholerae O1 lacking known toxins, including CT.

Materials and methods

Bacterial strains

Seven strains of V. cholerae O1 (GP5, GP6, GP12, GP71, GP156, V96 and VC20) were selected from our culture collection and used in this study. The choice of strains was dictated by the results of previous studies [15, 16] and the strains were selected to represent as many combinations as possible of the virulence genes as shown in Table 1. All the strains were biochemically characterised, including determination of polymyxin B sensitivity by methods described previously [17]. The strains were maintained as stabs in nutrient agar at 4°C. Streaks were run concurrently when the culture supernates of the test strains were assayed by the bead-ELISA.

Detection of CT production

The test strains were grown in yeast extract peptone (YEP) [18] with shaking (120 rpm) at 37°C. The culture supernates of the V. cholerae O1 strains were examined for the presence of CT by a highly sensitive bead-ELISA [19]. Various dilutions of purified CT (Sigma) and uninoculated medium (negative control) were run concurrently when the culture supernates of the test strains were assayed by the bead-ELISA.

Production of haemolysin

Brain Heart Infusion Broth (BHIB; Difco Laboratories, MI, USA) with NaCl 0.5% was used to grow the strains for determination of El Tor haemolysin production [20]. The strains were grown in 2 ml of BHIB in test tubes (10 × 13 cm) and also in 50 ml of BHIB contained in 250-ml Erlenmeyer flasks. Washed rabbit erythrocytes diluted to a final concentration of 1% in 10 mM phosphate buffer (pH 7.0) containing NaCl 1.3% were mixed with an equal volume of the culture supernate and incubated at 37°C for 1 h. The mixture was then centrifuged at 1000 g for 5 min and the amount of released haemoglobin in the supernate was measured spectrophotometrically at 540 nm.

Polymerase chain reaction assay

A multiplex polymerase chain reaction (PCR)-based assay was used to determine the presence of ctxA (encoding the enzymic subunit of CT) and tcpA (encoding the major structural subunit of TCP), while a single-format PCR assay was used for toxR (encoding the transcription activator ToxR), zot (encoding Zot) and ace (encoding Ace). Primer pairs were used to detect the following: a 301-bp fragment of ctxA [21], a 471-bp fragment of El Tor variant of tcpA, a 671-bp fragment of classical variant of tcpA [22], a 990-bp fragment of toxR [23], a 243-bp fragment of zot and a 284-bp fragment of ace [24].

The following were added to each 100 μl of PCR mixture: 10 μl of 10× PCR buffer (100 mM TAPS, pH 8.8; 500 mM KCl; 15 mM MgCl₂ and gelatin 0.1%), 2 μl each of 2.5 mM dATP, dGTP, dCTP and dTTP, 100 pmol each of the primers and 2 U of Taq DNA polymerase (Takara Shuzo Co. Ltd, Japan). PCR was carried out in 0.5-ml thin-walled PCR tubes (Midwest, USA), with 42 μl of the PCR mixture described above and 8 μl of a Luria broth culture of the test strain, overlaid with mineral oil, heated at 94°C for 5 min and processed in a DNA thermal cycler (Biometra, Göttingen, Germany). PCR amplification was performed for 30 cycles and the cycling conditions for ctxA, tcpA and toxR were: denaturation at 94°C for 1 min, annealing at 60°C for 1.5 min and extension at 72°C for 1.5 min; for zot and ace the time for annealing and extension was 1 min each. A reagent blank (containing all the components of the reaction mixture and Luria broth instead of broth containing template DNA) and two V. cholerae O1 strains – one belonging to the El Tor biotype (VC20) and the other to the classical biotype (569B) – from our culture collection were run as controls. Amplified products from the PCR were electrophoresed on agarose 1.5% gels and were stained with ethidium bromide. φX174-
Hae III (Takara Shuzo Co. Ltd) mol. wt marker was run concurrently.

**Rabbit ileal loop assay**

Challenge doses were prepared by growing the bacterial strains to mid-exponential phase in Tryptic Soy Broth (Difco) at 37°C followed by low speed centrifugation and resuspension to c. 10⁸ bacteria/ml in phosphate-buffered saline (PBS, pH 7.2) for introduction into the ligated loops. Culture filtrates of the test strains, grown in various broths – i.e., AKI [25], YEP [18], BHIB, casamino-acid-Yeast Extract medium with lincomycin 90 mg/L (CAYE-L) and syncase medium [26] – were obtained from the supernate which remained after centrifugation of the culture at 6000 g for 20 min in the cold.

Surgical procedures for the ligated intestinal loop test according to the method described by De and Chatterje [27] were carried out on outbred New Zealand White rabbits of either sex weighing c. 2.0–2.5 kg. Briefly, laparotomy was performed on animals anaesthetised by intramuscular injection of ketamine (35 mg/kg body weight) and xylazine (5 mg/kg body weight). The small intestine was withdrawn and ligated at a distance of c. 10 cm from the ileocecal region. Eight intestinal loops of 6–10 cm, separated by uninoculated segments of 1–2 cm, were made in each animal. Test loops were inoculated with 1-ml volumes of bacterial suspensions or culture supernates. Negative control loops received either PBS alone or the uninoculated medium, while the positive control loops received bacterial suspension of the prototype V. cholerae 01 biotype El Tor strain (VC20). Loops were replaced in the peritoneal cavity in the original position. Eight intestinal loops of 6–10 cm, separated by uninoculated segments of 1–2 cm, were made in each animal. Test loops were inoculated with 1-ml volumes of bacterial suspensions or culture supernates. Negative control loops received either PBS alone or the uninoculated medium, while the positive control loops received bacterial suspension of the prototype V. cholerae 01 biotype El Tor strain (VC20). Loops were replaced in the peritoneal cavity in the original position and the peritoneum was closed. After 18–20 h, the animal was killed with sodium pentobarbital and the abdomen was re-opened. The loops were taken out and the length of each loop in cm and the volume and the extent of the fluid accumulation (FA) was expressed as ml of fluid accumulation.

**Ussing chamber experiments**

The test strains were cultured in 50 ml of AKI, YEP, BHIB, CAYE-L and syncase medium and incubated at 37°C with shaking (200 rpm). The cell-free culture filtrate obtained by centrifugation at 5000 g for 20 min and passage through a 0.22 μm pore size disposable filter was lyophilised and resuspended in 15 ml of sterile double-distilled water. Ussing chamber experiments were performed with segments of small intestine from male adult New Zealand white rabbits (body weight 1.5–2.5 kg). Rabbits were anaesthetised by intramuscular injection of ketamine (35 mg/kg body weight) and xylazine (5 mg/kg body weight) and killed by air embolism. A 15-cm segment of distal ileum was teased loose from the mesentery, flushed free of intestinal contents with Ringer’s solution (115 mM NaCl, 25 mM NaHCO₃, 2.5 mM K₂HPO₄, 0.4 mM KH₂PO₄, 1.2 mM MgCl₂, 1.2 mM CaCl₂), opened along the mesenteric border, and stripped of muscular and serosal layers. Sheets of mucosa were then mounted on prongs in Lucite Ussing chambers with an aperture of 1.13 cm² and bathed by freshly prepared Ringer’s solution which was maintained at 37°C with water-jacketed reservoirs connected to a constant temperature circulating pump and gassed with O₂ 95% and CO₂ 5%. The potential difference and short circuit current (Isc) were measured. The potential difference was allowed to reach a steady state. A 400 μl volume of test supernate was added to either side of the mucosa. The changes in Isc were recorded at 5-min intervals for the first reading and then at 15-min intervals up to 110 min. The non-parametric Kruskal-Wall’s test was applied to determine the statistical difference in variation in conductance of culture supernate of strains grown in different media.

**Results**

**Phenotypic and genotypic traits of the strains**

Of the seven strains included in this study, five were wild-type mutants that lacked all the virulence genes in the CTX virulence cassette (GP5, GP6, V96), or contained internal deletion within the CTX virulence cassette (GP71), or, in addition to an intact CTX virulence cassette, also carried sto and produced NAGST-like toxin (GP156) [16, 28] as shown in Table 1. The prototype V. cholerae 01 strain of the El Tor biotype was represented by strain VC20 [29] and the classical biotype was represented by strain GP12, and these were used as the positive controls. Strains GP156, GP12 and VC20 produced CT when examined by the bead-ELISA (Table 1) and yielded the 301-bp ctaA amplicon; the other strains were negative for the factors in both assays. All the strains yielded the 990-bp fragment of toxR amplicon whereas only four of the seven strains (GP12, GP71, GP156, and VC20) yielded the 243-bp zot amplicon and 284-bp ace amplicon. Apart from strain GP6, all strains yielded the tcpA amplicon. In five strains (GP5, V96, GP71, GP156 and VC20), the tcpA amplicon was 471 bp, which was consistent with the El Tor variant of the tcpA gene. The size of the tcpA amplicon of strain GP12 was 621 bp, suggesting that the strain belonged to the classical biotype. Also, strain GP12 was sensitive to polymyxin B, a trait which is consistent with the classical biotype. All the strains carried the structural gene (hlyA) for the El Tor haemolysin; only strains GP71 and GP156 produced El Tor haemolysin when grown in 50 ml cultures, but none of these strains produced the haemolysin when grown in 2-ml cultures. Strain VC20, the prototype V. cholerae 01 strain of El Tor biotype, is genotypically hlyA-positive, but shows no haemolytic activity in vitro and this is consistent...
Table 1. Genotypic and phenotypic traits of the *V. cholerae* O1 strains examined in this study

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Place of isolation</th>
<th>Year of isolation</th>
<th>Serotype</th>
<th>hlyA</th>
<th>toxR</th>
<th>ctxA</th>
<th>zot</th>
<th>ace</th>
<th>tcpA</th>
<th>E1 Tor</th>
<th>Classical</th>
<th>Heat-stable enterotoxin gene (sto)*</th>
<th>CT production (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP5</td>
<td>Macao</td>
<td>1970</td>
<td>Inaba</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GP6</td>
<td>Macao</td>
<td>1970</td>
<td>Inaba</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V96</td>
<td>India</td>
<td>1990</td>
<td>Ogawa</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GP12</td>
<td>India</td>
<td>1971</td>
<td>Ogawa</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GP71</td>
<td>India</td>
<td>1974</td>
<td>Ogawa</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GP156</td>
<td>Australia</td>
<td>1979</td>
<td>Inaba</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VC20</td>
<td>Calcutta</td>
<td>(India)</td>
<td>Ogawa</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Data on sto from references [16, 28].

With the characteristics of recent El Tor strains which are non-haemolytic or weakly haemolytic.

*Enterotoxicity of whole cells examined by rabbit ileal loop test*

Of the whole cell preparations of the seven strains examined, four (GP5, GP6, GP71 and V96) were non-secretogentic or the amount of fluid accumulated in the test loops of rabbits was negligible; one strain (GP12) was moderately secretogentic and two (GP156 and the positive control strain VC20) induced fluid accumulation consistent with that of a fully toxigenic strain of *V. cholerae* (Table 2).

*Enterotoxigenicity of strains grown in different broths in the rabbit ileal loop test*

Culture supernates of the test strains grown in five different broth media showed variations in their ability to induce fluid accumulation in the ligated loop between media, but followed a general overall pattern that reflected the trend shown with whole cells (Table 2). Again, the secretogenticity of the test strains in ascending order was V96, GP71, GP5, GP6, GP12, VC20 and GP156. A revealing finding was that, of the media used, AKI and BHIB appeared to support the production of an unknown secretogenic factor, because all the test strains showed higher FA ratios than with other media. This included strains GP5, GP6 and V96, which by definition are non-toxigenic *V. cholerae* O1 and should have been benign in the rabbit ligated loop.

*Enterotoxigenicity of strains in Ussing chambers*

Culture supernate of strains of *V. cholerae* O1 grown in different media were added to rabbit ileal tissue mounted between lucite chambers maintained under conditions of ionic, osmotic and electrical equilibrium to investigate whether the supernates induced an increase in Isc. A general pattern was observed, with the fully toxigenic strain VC20 inducing the largest increase in Isc irrespective of the medium used (Table 3). Interestingly, the other fully toxigenic strain (GP156) did not induce changes in Isc consistent with that of the presence of CT and NAG-ST in all media. In contrast, strain GP12 – which is a fully toxigenic classical strain – evoked higher Isc changes than strain GP156, and was more or less similar to strain VC20 in this respect. The broth medium used appeared to effect changes in Isc with strain V96, the non-toxigenic strain, showing fairly high Isc changes in most media except syncase. Again, compared with the toxigenic strains, the non-toxigenic strains GP5 and GP6 showed

Table 2. Fluid accumulating ability of the strains of *V. cholerae* O1 in rabbit ligated ileal loop

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Fluid accumulation ratio (ml/cm)</th>
<th>Supernate of strain grown in†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole cells*</td>
<td>AKI</td>
</tr>
<tr>
<td>GP5</td>
<td>0</td>
<td>0.49</td>
</tr>
<tr>
<td>GP6</td>
<td>0.10 (0.18)</td>
<td>0.55</td>
</tr>
<tr>
<td>V96</td>
<td>0.19 (0.23)</td>
<td>0.31</td>
</tr>
<tr>
<td>GP12</td>
<td>0.41 (0.14)</td>
<td>1.16</td>
</tr>
<tr>
<td>GP71</td>
<td>0</td>
<td>0.47</td>
</tr>
<tr>
<td>GP156</td>
<td>1.13 (0.19)</td>
<td>1.18</td>
</tr>
<tr>
<td>VC20</td>
<td>1.04 (0.18)</td>
<td>1.12</td>
</tr>
<tr>
<td>Medium</td>
<td>✸</td>
<td>0.09</td>
</tr>
</tbody>
</table>

*Values are mean (SE) of experiments performed in five rabbits.
†Values are mean of experiments performed in two rabbits.
lower changes in Isc over a period of time. Medium again played a role in changes in Isc with strain GP6 evoking hardly any change in Isc when grown in CAYE-L, but showing substantial changes in Isc, compared with the medium control, in all the other media used (Table 3).

The conductance values of culture supernates of different strains grown in different media are presented in Table 4. Conductance of a tissue is a useful index of the leakiness of the tissue – increased conductance indicating disruption of function of the tight junctions. Conductance was again influenced by the medium used and the least changes in conductance were observed in YEP and BHIB. For example, with the non-toxigenic strains (GP5, GP6, V96), increases in conductance indicating disruption of function of the tight junctions. Conductance was again influenced by the medium used and the least changes in conductance were observed in YEP and BHIB. For example, with the non-toxigenic strains (GP5, GP6, V96), increases in conductance were observed when they were grown in AKI medium as compared with YEP and BHIB. In CAYE-L and syncase media, the two non-toxigenic strains (GP5, GP6) did not evoke changes in conductance. This pattern in the changes of conductance was also consistent with all the other strains. However, the variation in conductance of the various strains grown in different media was not statistically significant.

Discussion

After more than 17 years of elegant and vigorous research on the development of oral recombinant vaccine strains for cholera, it has become clear that the pathogenesis of cholera is not as straightforward as previously perceived. From the human volunteer studies with recombinant oral cholera vaccine strains [12], the involvement of another toxin in addition to those currently recognised or the process of colonisation, or both, have been attributed to be the cause of the mild to moderate residual diarrhoea [1, 12]. Interest in the ancillary secretogenic factors was further stimulated by the clinical observation of cholera-like diarrhoea caused by V. cholerae belonging to the non-O1 non-O139 serogroups. Although the percentage of such patients hospitalised at the Infectious Diseases Hospital in Calcutta is usually <4.9% [30], it presented an enigma as to how strains of V. cholerae devoid of the CTX virulence cassette were still able to cause a clinical condition very similar to cholera. To add to the mystery, non-toxigenic strains of V. cholerae O1 associated with a cluster of cases of cholera were encountered recently in southern India [31]. All these events led to the design of the present study, to answer the basic question as to whether V. cholerae devoid of the CTX virulence cassette is enterotoxigenic.

Strains GP5, GP6 and V96 represented the non-toxigenic strains of V. cholerae O1 in this study. Genotypically, the three strains were very similar, except that strain GP6 did not possess tcpA. Whole cells of the three non-toxigenic strains were inert in the rabbit ileal loop assay, but culture filtrate of the three strains showed enterotoxic activity, especially

Table 3. Variation in Isc in Ussing chambers after addition of 400 μl of culture supernates of different wild-type V. cholerae O1 strains grown in AKI, YEP, BHIB, CAYE-L and syncase to both mucosal and serosal sides of rabbit intestinal tissue recorded after 35 min and 110 min

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Isc 35 min</th>
<th>Isc 110 min</th>
<th>Isc 35 min</th>
<th>Isc 110 min</th>
<th>Isc 35 min</th>
<th>Isc 110 min</th>
<th>Isc 35 min</th>
<th>Isc 110 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP5</td>
<td>22 (12)</td>
<td>33 (8)</td>
<td>31 (21)</td>
<td>44 (4)</td>
<td>32 (23)</td>
<td>54 (11)</td>
<td>42 (20)</td>
<td>46 (13)</td>
</tr>
<tr>
<td>GP6</td>
<td>48 (17)</td>
<td>49 (12)</td>
<td>47 (17)</td>
<td>56 (3)</td>
<td>49 (29)</td>
<td>48 (16)</td>
<td>13 (9)</td>
<td>27 (12)</td>
</tr>
<tr>
<td>GP12</td>
<td>52 (15)</td>
<td>76 (11)</td>
<td>33 (15)</td>
<td>67 (0)</td>
<td>29 (17)</td>
<td>79 (24)</td>
<td>32 (14)</td>
<td>49 (9)</td>
</tr>
<tr>
<td>GP71</td>
<td>19 (13)</td>
<td>39 (9)</td>
<td>38 (16)</td>
<td>56 (4)</td>
<td>27 (26)</td>
<td>60 (28)</td>
<td>33 (21)</td>
<td>55 (26)</td>
</tr>
<tr>
<td>GP156</td>
<td>20 (2)</td>
<td>56 (2)</td>
<td>45 (24)</td>
<td>81 (26)</td>
<td>11 (3)</td>
<td>49 (4)</td>
<td>48 (16)</td>
<td>85 (28)</td>
</tr>
<tr>
<td>V96</td>
<td>35 (10)</td>
<td>63 (6)</td>
<td>23 (13)</td>
<td>52 (10)</td>
<td>31 (15)</td>
<td>69 (7)</td>
<td>30 (13)</td>
<td>57 (19)</td>
</tr>
<tr>
<td>VC20</td>
<td>39 (23)</td>
<td>80 (15)</td>
<td>22 (12)</td>
<td>91 (23)</td>
<td>43 (29)</td>
<td>75 (7)</td>
<td>56 (37)</td>
<td>90 (30)</td>
</tr>
<tr>
<td>Medium</td>
<td>14 (6)</td>
<td>17 (5)</td>
<td>11 (6)</td>
<td>21 (5)</td>
<td>23 (4)</td>
<td>11 (3)</td>
<td>5 (8)</td>
<td>7 (7)</td>
</tr>
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</table>

Values are mean (SEM) of four experiments.

Table 4. Variations in conductance in Ussing chambers induced by culture supernates of the various V. cholerae O1 strains recorded after 95 min

<table>
<thead>
<tr>
<th>Medium</th>
<th>GP5</th>
<th>GP6</th>
<th>V96</th>
<th>GP12</th>
<th>GP71</th>
<th>GP156</th>
<th>VC20</th>
<th>Medium control</th>
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<tbody>
<tr>
<td>AKI</td>
<td>66 ± 5</td>
<td>83 ± 33</td>
<td>62 ± 14</td>
<td>58 ± 0</td>
<td>30 ± 8</td>
<td>50 ± 8</td>
<td>63 ± 24</td>
<td>37 ± 16</td>
</tr>
<tr>
<td>YEP</td>
<td>31 ± 6</td>
<td>36 ± 1</td>
<td>45 ± 15</td>
<td>50 ± 0</td>
<td>35 ± 0</td>
<td>44 ± 0</td>
<td>39 ± 3</td>
<td>31 ± 8</td>
</tr>
<tr>
<td>BHIB</td>
<td>36 ± 5</td>
<td>56 ± 17</td>
<td>47 ± 8</td>
<td>45 ± 8</td>
<td>60 ± 5</td>
<td>32 ± 6</td>
<td>45 ± 11</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>CAYE-L</td>
<td>48 ± 21</td>
<td>30 ± 6</td>
<td>35 ± 14</td>
<td>30 ± 12</td>
<td>25 ± 8</td>
<td>50 ± 14</td>
<td>56 ± 16</td>
<td>34 ± 21</td>
</tr>
<tr>
<td>Syncase</td>
<td>45 ± 5</td>
<td>45 ± 20</td>
<td>59 ± 18</td>
<td>60 ± 15</td>
<td>48 ± 5</td>
<td>47 ± 18</td>
<td>76 ± 15</td>
<td>40 ± 12</td>
</tr>
</tbody>
</table>

Values are mean ± SE of four experiments.
When grown in AKI and BHIB. Likewise, a medium-dependent increase in I sc and tissue conductance were observed in the Ussing chambers experiment. Taken together, it could be summarised that the three non-toxigenic strains possessed a medium-dependent enterotoxic factor. It appears that some component in AKI and BHIB triggers the production of the elusive secretogon. It is pertinent to mention here that certain clinical strains of V. cholerae non-O1 produced an extracellular factor when grown in AKI medium that evoked a rapid and dramatic cytotoxic response which manifested as cell rounding of CHO and HeLa cells without accompanying membrane damage [32]. The purified cytotoxin has shown impressive enterotoxic activity in the ligated ileal loop and is reportedly present in 81.3 and 100% of the toxigenic and non-toxigenic strains, respectively, of V. cholerae O1 [33].

The magnitude of difference in I sc of the strains grown in different media was more or less similar in the Ussing chambers, although strains grown in AKI and BHIB showed greater fluid accumulation in the rabbit ileal loop test. This appears to be contradictory, but there could be a reason for this. The fluid accumulation in rabbit ileal loop is the end-product of the manifestation of an enterotoxic factor, while changes in I sc reflect either ion secretion (increase in I sc and potential difference) or perturbation in the paracellular junction (increase in tissue conductance) of ileal tissue. In other words, the results of the Ussing chamber experiments give an idea of the possible mechanism involved in inducing the secretogenic effect. In this case, it appears that the increased fluid accumulation in rabbit ileal loop observed with strains grown in AKI and BHIB may not be caused by an increase in I sc, but by a mechanism other than this which needs to be investigated.

The inertness of the whole cells of non-toxigenic V. cholerae O1 in the rabbit ileal loop suggests that these strains possess an enterotoxic potential, but need appropriate external signals to stimulate production of the factor. It is now known that various environmental signals such as osmolarity, pH, temperature, amino acids, CO2 and iron control the expression of co-ordinately regulated virulence determinants in V. cholerae and other bacteria (reviewed by Mekalanos) [34]. An alternative explanation is that the non-toxigenic strains probably did not colonise and grow well in the rabbit ileal loops as compared with their toxigenic counterparts. Previous studies have documented that in-vivo growth of V. cholerae is greater for fully toxigenic strains than for poorly toxigenic strains [35, 36, 37]. It has been suggested that intestinal fluid secretion stimulated by CT creates an improved environment for bacterial multiplication [38]. Evidently, the supernates of non-toxigenic strains of V. cholerae O1 examined in this study contain material that manifests enterotoxic activity in rabbit ileal loops and in Ussing chambers when grown in an appropriate medium. We are unable to explain why whole cells of GP12 (the classical biotype strain) with an intact CTX virulence cassette were unable to evoke as much fluid accumulation as the prototype El Tor biotype strain, although culture supernate of this strain yielded data consistent with that of a toxigenic strain in rabbit ileal loops and in Ussing chambers.

Strain GP71 was chosen to assess the contributory effect of the natural deletion of the ctxA gene located within the CTX virulence cassette. The ctxA gene was clearly the most important constituent gene in the CTX virulence cassette in terms of evoking a secretory response. Strain GP71 was not secretogenic when whole cells were introduced in the ileal loop, but secretogenic when supernates of the strain grown in broth were introduced into the ileal loop. Although these results appear conflicting, it is possible that in the broth some environmental signal may have triggered the production of an enterotoxic factor which did not happen when the cells were introduced directly into the rabbit ileal loop.

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