Investigation of the Roles of Toxin-Coregulated Pili and Mannose-Sensitive Hemagglutinin Pili in the Pathogenesis of *Vibrio cholerae* O139 Infection

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In this study, adult volunteers were fed tcpA and mshA deletion mutants of *V. cholerae* O139 strain CVD 112 to determine the role of toxin-coregulated pili (TCP) and mannose-sensitive hemagglutinin (MSHA) in intestinal colonization. Eight of 10 volunteers who received CVD 112 or CVD 112 ΔmshA shed the vaccine strains in their stools; the geometric mean peak excretion for both groups was 1.4 × 10^8 CFU/g of stool. In contrast, only one of nine recipients of CVD 112 ΔtcpA shed vibrios in his stool (*P* < 0.01); during the first 24 h after inoculation, 3 × 10^7 CFU/g was recovered from this volunteer. All recipients of CVD 112 and 8 (80%) of the recipients of CVD 112 ΔmshA developed at least a fourfold rise in vibriocidal titer after immunization. In contrast, only one (11%) of the nine recipients of CVD 112 ΔtcpA developed a fourfold rise in vibriocidal titer (*P* < 0.01). We conclude that TCP are an important colonization factor of *V. cholerae* O139 and probably of El Tor *V. cholerae* O1. In contrast, MSHA does not appear to promote intestinal colonization in humans.

Adherence of *Vibrio cholerae* to the intestinal mucosa might be mediated by different mechanisms, depending on the biotype (classical or El Tor) and serotype (O1 and O139). It is clear that colonization of strains of *V. cholerae* O1 of the classical biotype is mediated by toxin-coregulated pili (TCP), which are encoded by tcpA (20). The importance of TCP in the pathogenesis of cholera was demonstrated in volunteer studies. Classical Ogawa *V. cholerae* O1 strain 395 with deletions in tcpA did not cause diarrhea, did not colonize the duodenal fluids or stools of volunteers, and did not induce vibriocidal or antitoxic immune responses (6, 7).

The tcpA gene is also present in El Tor biotype *V. cholerae* O1 strains (9, 16). In addition, most, if not all, strains of El Tor *V. cholerae* O1 express another pilus, called mannose-sensitive hemagglutinin (MSHA) (10). Mutations in tcpA and mshA have been constructed, and the strains have been studied with infant mice. The colonization of the mshA-deleted El Tor *V. cholerae* O1 strain was no different from that of the wild type; in contrast, the El Tor strain with deletions in tcpA was markedly reduced in its ability to colonize infant mice (1, 2, 21).

The roles of these two pili in mediating protective immunity have been studied with animals. Antibodies against classical TCP have provided variable protection against *V. cholerae* El Tor in mice (1, 15, 17, 18, 22). The inconsistent protection of anti-TCP antibodies is likely explained by the sequence differences between El Tor and classical TCP; these proteins show 82% identity (16). The differences in protection mediated by TCP antibodies may be due to the difference in the specificities of anti-classical TCP serum and anti-El Tor TCP serum.

The mechanism of colonization of *V. cholerae* O139 has not been established. *V. cholerae* O139 strains are closely related to El Tor strains of the O1 group (3, 5, 8, 23), so one might expect that colonization factors of El Tor O1 strains would also be important in O139 strains. The gene for TCP pilin is present in O139 strains, and the amino acid sequence is identical to that of El Tor O1 strains (16). Mutants of *V. cholerae* O139 strain M03 with deletions in tcpA and mshA have been constructed; in colonization competition studies with the wild type, the tcpA deletion mutant was markedly decreased in colonization (21). In contrast, the ΔmshA mutant was somewhat better able to compete for colonization of mice. In an independent study, an O139 strain with deletions in mshA had no competitive advantage (1). These data suggest that TCP are essential for the colonization of infant mice with *V. cholerae* O139 and that MSHA does not appear to have a significant role.

The purpose of this study was to determine the role of tcpA and mshA in the intestinal colonization of volunteers given *V. cholerae* O139 vaccine strain CVD 112 modified by deletions in tcpA and mshA (designated strains KHT47 and KHT37, respectively). CVD 112 is a derivative of *V. cholerae* O139 strain A1837, designed as a vaccine candidate by deletions in genes for cholera toxin A subunit (ctxA), zonula occludens toxin, accessory cholera enterotoxin, and core encoded pilin, which are on the bacteriophage CTXΦ (24). In the volunteer study described here, we chose to use CVD 112 to avoid the risks of dehydrating diarrhea in volunteers while still addressing questions about colonization.

**MATERIALS AND METHODS**

**Clinical study design.** Healthy adult volunteers were educated about cholera and the requirements of the protocol, and informed, written consent was obtained from each volunteer. Prospective volunteers were carefully screened to ensure that they were in excellent physical and mental health. Screening consisted of a medical history, physical examination, interview by a clinical psychologist, and a battery of blood tests.

A group of 29 inpatient volunteers was admitted to the research isolation ward, located in the University of Maryland Hospital. They were randomized to receive the following with sodium bicarbonate buffer: (i) 1 × 10^7 to 2 × 10^7 CFU of *V. cholerae* O139 vaccine strain CVD 112 (*n* = 10), (ii) 1 × 10^7 to 2 × 10^7 CFU of *V. cholerae* O139 strain CVD 112 ΔtcpA (designated KHT47) (*n* = 9), or (iii)
$1 \times 10^7$ to $2 \times 10^7$ CFU of *V. cholerae* O139 strain CVD 112 *ΔmshA* (designated KHT37) (*n* = 10).

Daily clinical observations were made in which symptoms were recorded. Volunteers who developed diarrhea were given oral rehydration (with World Health Organization glucose-electrolyte solution) after each loose stool. Volunteers were observed for 4 days and then treated with tetracycline (500 mg orally every 6 h for four doses), followed by a single oral dose of doxycycline. They were discharged from the isolation ward at day 7 after ingestion of vibrios.

From the time of admission, volunteers collected every bowel movement in plastic containers. After collection of a stool, the contents of the stool container were inspected and graded for consistency of the stool according to five grades: grade 1, firm; grade 2, soft; grade 3, thick liquid; grade 4, opaque watery; and grade 5, rice water. Grades 1 and 2 are variations of normal stools, while grades 3 to 5 are considered abnormal.

To culture vibrio from the proximal small intestine (the critical site of host-bacterium interaction), volunteers ingested gelatin-encapsulated string devices (Enterotest) approximately 20 and 44 h after ingestion of *V. cholerae* as previously described (7). Blood was collected before and 11 and 28 days after ingestion of vibrios in his stool (Table 1).

**RESULTS**

**Clinical and bacteriologic results.** Mild diarrhea occurred in 3 (30%) of 10 recipients of $10^7$ CFU of CVD 112, 5 (50%) of 10 recipients of $10^7$ CFU of CVD 112 *ΔmshA*, and in none of 9 recipients of $10^7$ CFU of CVD 112 *ΔtcpA* (Table 1). These rates of diarrhea among recipients of CVD 112 and CVD 112 *ΔmshA* were similar to those previously observed among recipients of similar doses of CVD 112 (24).

Eight of 10 volunteers who received CVD 112 or CVD 112 *ΔmshA* shed the vaccine strains in their stools; the geometric mean peak excretion for both groups was $1.4 \times 10^5$ CFU/g of stool (Table 1). This rate of shedding is similar to that observed after ingestion of the wild-type parent strain, AI1837 (19). In contrast, only one of nine recipients of CVD 112 *ΔtcpA* shed vibrios in his stool (*P* < 0.01); during only the first 24 h after inoculation, $3 \times 10^5$ CFU/g was recovered from this volunteer. The duodenal fluid cultures gave a similar pattern (Table 1). The numbers of organisms recovered from intestinal fluid were similar in recipients of CVD 112 and CVD 112 *ΔmshA* (1.6 $\times 10^3$ and 3.6 $\times 10^3$, respectively).

**Immune responses.** *V. cholerae* O139 stimulates meager titers of vibriocidal antibodies after wild-type infection, compared to the titers stimulated by *V. cholerae* O1, probably due to the presence of the capsule on O139 strains (13, 14). All recipients of CVD 112 and eight (80%) of the recipients of CVD 112 *ΔmshA* had seroconverted by day 11 after challenge with AI1837 (19). In contrast, only one of nine recipients of CVD 112 *ΔtcpA* had detectable vibriocidal antibodies, and the geometric mean optical density for IgG anti-Cholera toxin antibody was 0.04 (0/9) versus 0.43 (5/10) for recipients of CVD 112 (Table 1). The difference in antibody titers is significant by Student’s *t*-test (*P* < 0.01). The geometric mean peak optical density for IgG anti-Cholera toxin antibody and IgG anti-TCP antibody was 1.0 (0.8) versus 0.78 (2/10) (Table 1).

**Statistical analysis.** Rates of vibriocidal antibody conversion were compared by Fisher’s exact test. Comparison of antibody titers were performed on log-transformed reciprocal titers by Student’s *t*-test.

#### TABLE 1. Clinical and bacteriologic responses in volunteers who received 10⁷ CFU of *V. cholerae* O139 strain CVD 112, KHT37 (CVD 112 *ΔmshA*), or KHT47 (CVD 112 *ΔtcpA*).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diarrhea attack rate (%)</th>
<th>Mean diarrheal stool vol (ml)</th>
<th>Rate (%) of vibrio excretion</th>
<th>Geometric mean peak no. of organisms/g of stool</th>
<th>Rate (%) of positive duodenal fluid cultures</th>
<th>Geometric mean no. of organisms recovered from duodenal fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVD 112</td>
<td>3/10 (30)</td>
<td>693</td>
<td>8/10 (80)</td>
<td>$1.4 \times 10^3$</td>
<td>0/9 (0)</td>
<td>3/8 (38)</td>
</tr>
<tr>
<td>KHT37 (CVD 112 <em>ΔmshA</em>)</td>
<td>5/10 (50)</td>
<td>529</td>
<td>8/10 (80)</td>
<td>$1.4 \times 10^2$</td>
<td>2/9 (22)</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>KHT47 (CVD 112 <em>ΔtcpA</em>)</td>
<td>0/9 (0)</td>
<td>0</td>
<td>1/9 (11)</td>
<td>$3 \times 10^2$</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

#### TABLE 2. Immune responses in volunteers who received 10⁷ CFU of *V. cholerae* O139 strain CVD 112, KHT37 (CVD 112 *ΔmshA*), or KHT47 (CVD 112 *ΔtcpA*).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vibriocidal<em>α</em> antibody seroconversion rate (%)</th>
<th>Geometric mean peak reciprocal vibriocidal antibody titer</th>
<th>IgG anti-cholera toxin antibody seroconversion rate (%)</th>
<th>Geometric mean change in optical density for IgG anti-cholera toxin antibody</th>
<th>IgG anti-TCP antibody seroconversion rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVD 112</td>
<td>10/10 (100)</td>
<td>121</td>
<td>10/10 (100)</td>
<td>0.78</td>
<td>2/10 (20)</td>
</tr>
<tr>
<td>KHT37 (CVD 112 <em>ΔmshA</em>)</td>
<td>8/10 (80)</td>
<td>106</td>
<td>8/10 (80)</td>
<td>0.63</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td>KHT47 (CVD 112 <em>ΔtcpA</em>)</td>
<td>1/9 (11)</td>
<td>29*</td>
<td>0/9 (0)*</td>
<td>0.04</td>
<td>0/9 (0)</td>
</tr>
</tbody>
</table>

*α* Vibriocidal responses were measured against *V. cholerae* O139 strain 2L, an encapsulated mutant of strain AI1837, the parent strain of CVD 112.

* Number with result/number tested.

$P = 0.12$ and $0.02$ (Fisher’s exact tests) versus rates in CVD 112 and CVD 112 *ΔmshA* recipients, respectively.

$P < 0.01$ (Fisher’s exact test) versus rates in CVD 112 and CVD 112 *ΔmshA* recipients.

$P$ is not significant (Fisher’s exact tests).
CVD 112 ∆mshA developed a fourfold or greater rise in vibriocidal titer after immunization; the geometric mean peak titers were 121 and 106, respectively (Table 2). In contrast, only one (11%) of the nine recipients of CVD 112 ∆tcpA developed a fourfold rise in vibriocidal titer (P < 0.01, Student’s t tests comparing CVD 112 ∆tcpA responses to CVD 112 and CVD 112 ∆mshA responses). This volunteer was the one who shed small numbers of CVD 112 ∆tcpA in his stool for 1 day. The vibriocidal response in this volunteer was unusual, since it occurred on day 28 after vaccination and was not present on day 11 after vaccination, when the amount of vibriocidal antibody in U.S. volunteers usually peaks (4). None of the recipients of CVD 112 ∆tcpA developed anti-cholera toxin antibody, while all of the recipients of CVD 112 and 80% of the recipients of CVD 112 ∆mshA developed anti-cholera toxin antibody (P < 0.001, Fisher’s exact tests, comparing CVD 112 ∆tcpA to either of the other two groups). IgG antibodies against TCP were detected in 2 of 10 recipients of CVD 112, 1 of 10 recipients of CVD 112 ∆mshA, and none of the recipients of CVD 112 ∆tcpA.

TOC have another function in bacterial physiology: the pilus is the receptor for a filamentous bacteriophage, CTXΦ, which encodes V. cholerae toxins (24). This surface structure, then, is possibly the ultimate virulence factor of V. cholerae, since TCP mediate infection of the bacterium with the phage, which in turn encodes cholera toxin, the factor responsible for cholera gravis. Our study shows that, without TCP, V. cholerae is thoroughly disarmed.

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