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Carol O. Tacket
University of Maryland at Baltimore

Ronald K. Taylor
Dartmouth College

Genevieve Losonsky
University of Maryland at Baltimore

Yu Lim
University of Maryland at Baltimore

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Tacket, Carol O.; Taylor, Ronald K.; Losonsky, Genevieve; and Lim, Yu, "Investigation of the Roles of Toxin-Coregulated Pili and Mannose-Sensitive Hemagglutinin Pili in the Pathogenesis of *Vibrio cholerae* O139 Infection" (1998). *Open Dartmouth: Peer-reviewed articles by Dartmouth faculty*. 990.
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Investigation of the Roles of Toxin-Coregulated Pili and Mannose-Sensitive Hemagglutinin Pili in the Pathogenesis of *Vibrio cholerae* O139 Infection

CAROL O. TACKET,^{1*} RONALD K. TAYLOR,² GENEVIEVE LOSONSKY,¹ YU LIM,¹
JAMES P. NATARO,¹ JAMES B. KAPER,¹ AND MYRON M. LEVINE¹

Center for Vaccine Development, Department of Medicine, University of Maryland School of
Medicine, Baltimore, Maryland 21201,¹ and Department of Microbiology,
Dartmouth Medical School, Hanover, New Hampshire 03755²

Received 29 May 1997/Returned for modification 23 September 1997/Accepted 26 November 1997

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 Δ *mshA* shed the vaccine strains in their stools; the geometric mean peak excretion for both groups was 1.4×10^5 CFU/g of stool. In contrast, only one of nine recipients of CVD 112 Δ *tcpA* shed vibrios in his stool ($P < 0.01$); during the first 24 h after inoculation, 3×10^2 CFU/g was recovered from this volunteer. All recipients of CVD 112 and 8 (80%) of the recipients of CVD 112 Δ *mshA* developed at least a fourfold rise in vibriocidal titer after immunization. In contrast, only one (11%) of the nine recipients of CVD 112 Δ *tcpA* 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 Δ *mshA* 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 AI1837, 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 CTX Φ (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 Δ *tcpA* (designated KHT47) ($n = 9$), or (iii)

* Corresponding author. Mailing address: Center for Vaccine Development, 685 West Baltimore St., Room 480, Baltimore, MD 21201. Phone: (410) 706-5328. Fax: (410) 706-4171. E-mail: ctacket@umppa1.ab.umd.edu.

TABLE 1. Clinical and bacteriologic responses in volunteers who received 10^7 CFU of *V. cholerae* O139 strain CVD 112, KHT37 (CVD 112 $\Delta mshA$), or KHT47 (CVD 112 $\Delta tcpA$)

Strain	Diarrhea attack rate ^a (%)	Mean diarrheal stool vol (ml)	Rate ^a of vibrio excretion (%)	Geometric mean peak no. of organisms/g of stool	Rate ^a of positive duodenal fluid cultures (%)	Geometric mean no. of organisms recovered from duodenal fluid
CVD 112	3/10 (30)	693	8/10 (80)	1.4×10^5	3/8 (38)	1.6×10^3
KHT 37 (CVD 112 $\Delta mshA$)	5/10 (50)	529	8/10 (80)	1.4×10^5	2/9 (22)	3.6×10^3
KHT47 (CVD 112 $\Delta tcpA$)	0/9 (0) ^b	0	1/9 (11) ^c	3×10^2	0/7 (0) ^d	0

^a Number with result/number tested.

^b $P = 0.12$ and 0.02 (Fisher's exact tests) versus rates in CVD 112 and CVD 112 $\Delta mshA$ recipients, respectively.

^c $P < 0.01$ (Fisher's exact test) versus rates in CVD 112 and CVD 112 $\Delta mshA$ recipients.

^d P is not significant (Fisher's exact tests).

1×10^7 to 2×10^7 CFU of *V. cholerae* O139 strain CVD 112 $\Delta mshA$ (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 vibrio for measurements of vibriocidal and anti-cholera toxin and anti-TCP antibodies.

Preparation and administration of strains CVD 112, KHT47, and KHT37. KHT47 and KHT37 were constructed as previously described (21). The strains have no growth defect as determined by in vitro growth competition studies with their *lacZ*-negative parent (21). The mutant strains and CVD 112 agglutinated with anti-O139 antiserum to the same extent. The motilities of CVD 112, KHT37, and KHT47 were normal and equivalent as assessed by motility agar plates (Luria-Bertani agar containing 0.3% agar). The preparation of inocula for administration to volunteers has been previously described (19). The strains were administered orally with NaHCO_3 . Two grams of NaHCO_3 was dissolved in 5 oz (150 ml) of distilled water. Volunteers drank 4 oz of the NaHCO_3 solution; 1 min later, they ingested the vibrio strain suspended in the remaining 1 oz of NaHCO_3 water. Volunteers had nothing to eat or drink for 90 min before and after vaccination.

Definition of diarrhea. Diarrhea was defined as the passage of two or more unformed (grades 3 to 5) stools over a 48-h period that equaled or exceeded 200 g or a single voluminous stool if it totaled 300 g or greater.

Bacteriology. All stools were plated directly onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar as well as inoculated into alkaline peptone water enrichment broth for overnight incubation before being plated onto TCBS. Suspicious colonies were agglutinated with specific antiserum. Up to three stools each day were cultured quantitatively to determine the number of vibrios per gram of stool.

Duodenal strings were tweezed with a sterile gloved hand to express duodenal fluid. This was quantitatively cultured as described above as well as inoculated

directly onto TCBS. In addition, the strings were inoculated into alkaline peptone water for overnight incubation before inoculation of plates of TCBS agar.

Immunology. Serum vibriocidal responses were determined against strain 2L, the unencapsulated mutant of AI1837, the parent strain of CVD 112 (13). Immunoglobulin G (IgG) antitoxin antibodies were measured by previously described methods (12). TCP antibodies were also measured by enzyme-linked immunosorbent assay as described previously (6) with purified TCP (18).

Statistical analysis. Rates of diarrhea and antibody conversion were compared by Fisher's exact tests. Comparisons of antibody titers were performed on log-transformed reciprocal titers by Student's *t* test.

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 $\Delta mshA$, and in none of 9 recipients of 10^7 CFU of CVD 112 $\Delta tcpA$ (Table 1). These rates of diarrhea among recipients of CVD 112 and CVD 112 $\Delta mshA$ 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 $\Delta mshA$ shed the vaccine strains in their stools; the geometric mean peak excretion for both groups was 1.4×10^5 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 $\Delta tcpA$ shed vibrios in his stool ($P < 0.01$); during only the first 24 h after inoculation, 3×10^2 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 $\Delta mshA$ (1.6×10^3 and 3.6×10^3 , 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

TABLE 2. Immune responses in volunteers who received 10^7 CFU of *V. cholerae* O139 strain CVD 112, KHT37 (CVD 112 $\Delta mshA$), or KHT47 (CVD 112 $\Delta tcpA$)

Strain	Vibriocidal ^a antibody seroconversion rate ^b (%)	Geometric mean peak reciprocal vibriocidal antibody titer	IgG anti-cholera toxin antibody seroconversion rate ^b (%)	Geometric mean change in optical density for IgG anti-cholera toxin antibody	IgG anti-TCP antibody seroconversion rate ^b (%)
CVD 112	10/10 (100)	121	10/10 (100)	0.78	2/10 (20)
KHT37 (CVD 112 $\Delta mshA$)	8/10 (80)	106	8/10 (80)	0.63	1/10 (10)
KHT47 (CVD 112 $\Delta tcpA$)	1/9 (11) ^c	29 ^d	0/9 (0) ^e	0.04	0/9 (0)

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

^b Number with result/number tested.

^c $P < 0.01$ (Fisher's exact tests) versus rate in recipients of either CVD 112 or KHT37 (CVD 112 $\Delta mshA$).

^d $P < 0.01$ (Student's *t* test) versus titer in recipients of either CVD 112 or KHT37 (CVD 112 $\Delta mshA$).

^e $P < 0.001$ (Fisher's exact tests) versus rate in recipients of either CVD 112 or KHT37 (CVD 112 $\Delta mshA$).

CVD 112 $\Delta mshA$ developed a fourfold or greater rise in vibriocidal titer after immunization; the geometric mean peak reciprocal titers were 121 and 106, respectively (Table 2). In contrast, only one (11%) of the nine recipients of CVD 112 $\Delta tcpA$ developed a fourfold rise in vibriocidal titer ($P < 0.01$, Student's t tests comparing CVD 112 $\Delta tcpA$ responses to CVD 112 and CVD 112 $\Delta mshA$ responses). This volunteer was the one who shed small numbers of CVD 112 $\Delta tcpA$ 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 $\Delta tcpA$ developed anti-cholera toxin antibody, while all of the recipients of CVD 112 and 80% of the recipients of CVD 112 $\Delta mshA$ developed anti-cholera toxin antibody ($P < 0.001$, Fisher's exact tests, comparing CVD 112 $\Delta tcpA$ 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 $\Delta mshA$, and none of the recipients of CVD 112 $\Delta tcpA$.

DISCUSSION

This clinical study clearly demonstrates the importance of TCP expression for *V. cholerae* O139 to colonize the intestine, cause diarrhea, and stimulate immune responses. Deletion in $tcpA$ resulted in the absence of diarrhea and marked decrease in colonization and immune responses in volunteers. Deletion in $mshA$ had no effect on the attack rate of diarrhea, the volume of diarrheal stool, or the numbers of vibrios recovered in duodenal fluid or stool. Our data suggest that MSHA is not necessary for colonization, and a role for MSHA in producing mild diarrhea associated with CVD 112 could not be demonstrated.

V. cholerae O139 is strikingly similar to biotype El Tor *V. cholerae* O1 and probably evolved recently from an El Tor strain (3, 5, 8). Although O139 strains express a polysaccharide capsule and have an altered lipopolysaccharide, they are similar to El Tor O1 strains by DNA sequences of crucial virulence factors and by multilocus enzyme electrophoresis. It is likely, therefore, that TCP are the critical colonization factor for El Tor O1, as they are for classical O1 strains (7).

This study emphasizes the importance of colonization for stimulating an immune response to *V. cholerae*. This finding has implications for the development of live attenuated vaccine strains, which should express TCP, and killed-whole-cell vaccines, which should contain TCP on the surfaces of classical and El Tor components, to stimulate protective immune responses. The very low (11%) vibriocidal seroconversion rate among recipients of KHT47 ($\Delta tcpA$) demonstrates that *V. cholerae* must express this antigen to stimulate serum vibriocidal antibodies, the best immune correlate of protection against cholera. These data also support the possibility that in humans, like in animals, strong mucosal immunity against the single antigen TCP could be adequate for protection against cholera by interfering with colonization and diarrheagenicity. Protection against another toxigenic enteric pathogen, *Escherichia coli*, appears to be mediated by anti-colonization factor responses (11). However, unlike *E. coli* colonization factors, TCP are only weakly immunogenic in humans after infection with live *V. cholerae* O1 (6) and after vaccination with O139 strain CVD 112, and so anti-TCP responses do not participate in natural immunity. It is possible that an immune response to TCP could be stimulated by presenting this antigen to mucosal immune sites with an appropriate carrier or with a mucosal adjuvant.

TCP have another function in bacterial physiology: the pilus is the receptor for a filamentous bacteriophage, CTX Φ , 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.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health contract NO1 AI-65299 to C.O.T. and by AI-25096 to R.K.T.

We acknowledge the excellent clinical care provided by the Center for Vaccine Development nursing staff. We are indebted to Kathy Palmer for excellent research coordination.

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Editor: J. R. McGhee