Brief Definitive Report

TOXIN, TOXIN-COREGULATED PILI, AND THE *toxR* REGULON ARE ESSENTIAL FOR *VIBRIO CHOLERAE* PATHOGENESIS IN HUMANS

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Pathogenic *Vibrio cholerae* O1 cause disease by colonizing the human small intestine, where they produce a potent enterotoxin (1). Many elements of the vibrio cell surface have been postulated to be involved in colonization, but an actual molecular component or morphological structure responsible for adherence to the human intestinal mucosa has not previously been conclusively demonstrated.

In the classical Ogawa *V. cholerae* O1 strain 395, a regulatory protein, ToxR, controls expression of cholera toxin (2). ToxR also regulates the expression of a rigid pilus, TcpA, in a coordinate manner with cholera toxin (3). Recently, *tcpA* and *toxR* insertion mutants of *V. cholerae* O1 were shown to be defective in colonization in suckling mice (3). To determine the role of TcpA in colonization of the human intestine, the bacteriology, and clinical and immunologic responses of healthy adult volunteers who ingested three Ogawa 395 mutants were assessed.

Materials and Methods

Bacterial Strains. Organisms used in this study are listed in Table I. EM of the parent strain 395 shows expression of TcpA filaments, each 5–7 nm in diameter, aggregating to form large bundles (3, 4, Fig. 1 *A*). Strain 395-N1, an isogenic mutant of 395, has an internal deletion in each of the two genes encoding the A subunit of cholera toxin (5). Strain JJM43, a derivative of 395-N1, carries an additional deletion in the *toxR* gene leading to markedly decreased expression of TcpA (3). Strain TCP2, also derived from 395-N1, carries an internal deletion in the *tcpA* gene that removes the coding sequence for amino acid residues 119–154 of the 199 residues making up mature TcpA subunits (6); consequently, TCP2 produces no pili of this type (Fig. 1 *B*).

Volunteers. Adult volunteers between the ages of 18 and 35 from the Baltimore metropolitan area participated in the studies. The methods of medical screening, care of the volunteers, and informed consent have been described (7, 8). Vaccination and challenge studies were carried out under quarantine in the 32-bed isolation ward maintained by the Center for Vaccine Development in the University of Maryland Hospital (Baltimore, MD). Protocols were reviewed by the Human Volunteer Research Committee of the University of Mary-

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TABLE I
Classical Vibrio cholerae O1 Ogawa Strains and Derivatives Tested in Volunteers

Strain	Parent	Genotype	TcpA phenotype
395		*ctx*A + B$^+$	Piliated
395-N1	395	*ctx*A − B$^+$	Piliated
JJM43	395-N1	*toxR* mutant	Decreased expression TcpA pili
TCP2	395-N1	*tcpA* mutant	Absent TcpA pili

land at Baltimore and the Clinical Research Sub-panel of the National Institute of Allergy and Infectious Diseases.

Bacteriology. Specimens of all stools were cultured both quantitatively and qualitatively. If no stool specimen was available in a 24-h period, a rectal swab was obtained. Stool and swabs were directly plated onto thiosulfate-citrate-bile salts-sucrose (TCBS) agar (BBL Microbiology Systems, Cockeysville, MD) and inoculated into alkaline peptone water enrichment broth. After overnight incubation, the enrichment broth was subcultured onto TCBS agar. Quantitative cultures were obtained by diluting 1.0 g (or 1.0 ml) of stool 10-fold serially in PBS (pH 7.2), inoculating 0.1 ml onto plates of TCBS agar, and counting colonies. Yellow colonies on TCBS agar were confirmed as *V. cholerae* O1 serotype Ogawa by agglutination with Ogawa typeing sera. Gelatin string capsule devices (Enterotest; HDC Corp., Mountain View, CA) were used to obtain duodenal fluid for culture (7). Fasting volunteers ingested string capsules at 6:00 a.m. on two consecutive days after vaccination. Strings were removed 4 h later, and the distal bile-stained portions were cultured as above. If the pH of the fluid obtained from the string was <6, indicating probable gastric origin, negative results were not reported.

Serology. Sera were collected from all volunteers before and 7, 21, and 28 d after ingestion of all strains. Vibriocidal antibody was measured by a microtiter technique (9). IgG antitoxin antibodies were measured by ELISA (10).

Results and Discussion

The clinical responses of volunteers to the attenuated strains are summarized in Table II. Strains TCP2 and 395-N1 (at 10^6 CFU) were compared in a double-blind fashion in the same study. Otherwise, the different strains or different doses of the same strain were tested at separate times. Diarrhea or fever (maximum temperature of 39°C) were documented in 3 of 13 volunteers receiving the higher dose of 395-N1 (10^8 viable organisms), and constitutional complaints were noted by approximately half of the recipients of either dose of this strain. Notable adverse reactions were not seen with strains JJM43 and TCP2.

Dramatic differences were seen in the bacteriologic responses to these strains (Table II). Recipients of 10^6 CFU of strains TCP2 or JJM43 never shed the respective organisms in their stool. Strain JJM43 was recovered from the stools of two of seven volunteers receiving 2×10^8 CFU, although duodenal string capsule cultures were negative. In contrast, strain 395-N1 colonized efficiently as determined by coprocultures and cultures of string capsule devices.

20 of 21 recipients of 395-N1 had prominent serum vibriocidal antibody responses, including all 13 volunteers who ingested 10^8 CFU (Table II). Although 9 of 12 JJM43 recipients also had seroconversions, the geometric mean vibriocidal titers were significantly lower than those seen after ingestion of 395-N1 (Table II). Likewise, the IgG cholera antitoxin responses elicited by JJM43 were significantly lower when compared with 395-N1 recipients. In distinction, none of eight recipients of TCP2 manifested significant rises in vibriocidal or antitoxin titers.

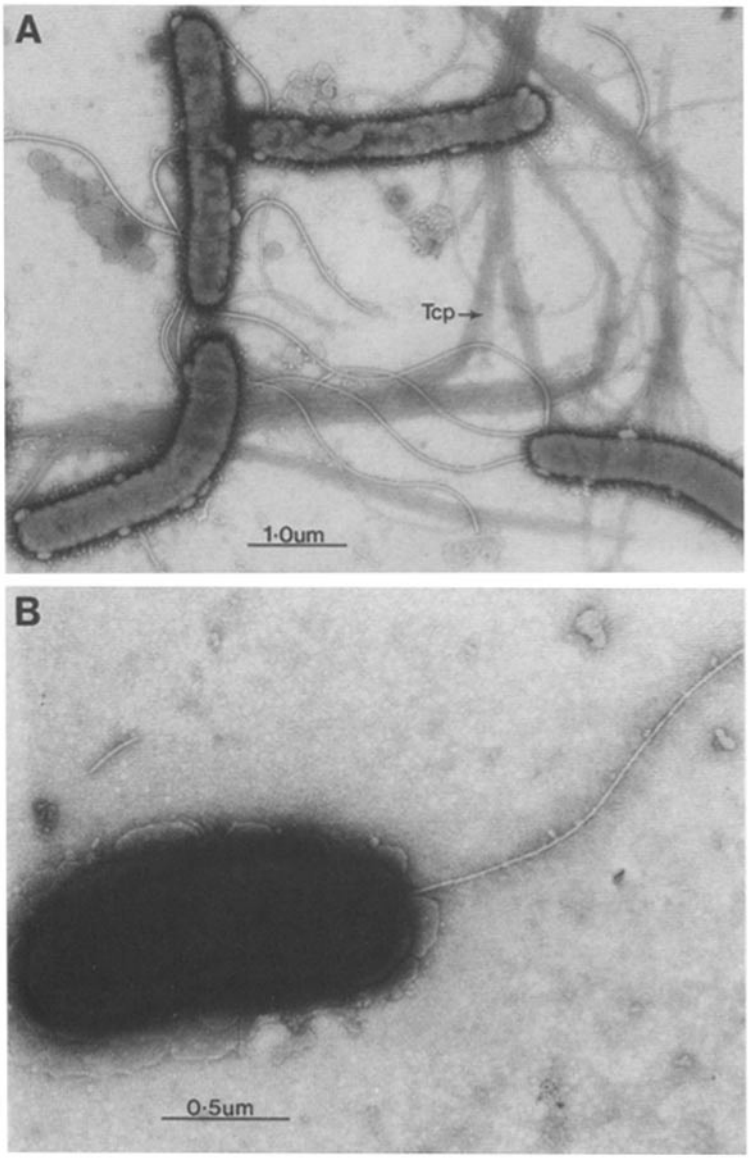

FIGURE 1. Electron photomicrographs showing *V. cholerae* O1 Ogawa 395 (*A*) and TCP2 (*B*) negatively stained with 1% phosphotungstic acid, pH 6.8. Bundles of TcpA are present only in strain 395 and are indicated by the arrow.

To assess the induction of protective immunity after immunization with 395-N1 or TCP2, four recipients of 395-N1, three who had received TCP2, and seven control volunteers were challenged with fully toxigenic *V. cholerae* O1 strain, Ogawa 395. Recipients of 395-N1 but not TCP2 were protected against clinical cholera (Table III).

The mechanisms by which *V. cholerea* O1 adheres to the enterocytes of the human small intestine has been the subject of intense research since it has obvious relevance to vaccine development (1). Animal models have previously implicated several com-

TABLE II

A Comparison of the Clinical and Immunologic Responses of Volunteers after Ingestion of Classical Ogawa 395 Derivatives TCP2, JJM43, and 395N1

Vibrio cholerae strain	Dose	Volunteers	Clinical response* (No. with:)			Bacteriology (No. with positive cultures)		Vibriocidal antibody		Antitoxin antibody‡	
			Diarrhea	Fever	More than one symptom§	Stool‖	String¶	No. with significant rises	Peak geometric mean titer	No. with significant rises	Mean peak titer
	CFU	*n*									*OD*
TCP2	10^6	8	0	0	1	0	0/7	0	52	0	0.10
JJM43	10^6	5	0	0	0	0	0/5	2	230(a)**	2	0.26(e)
JJM43	10^8	7	0	0	2	2 (5×10^3)	0/4	7	580(b)	4	0.64(f)
395N1	10^6	8	0	0	4	6 (5×10^4)	3/7	7	2,153(c)	6	0.67(g)
395N1	10^8	13	1	2	7	13 (6×10^5)	11/11	13	8,273(d)	13	0.95(h)

* Diarrhea was defined as two or more loose stools within 48 h totaling at least 200 ml in volume or a single loose stool >300 ml in volume. Fever was defined as oral temperature ≥37.8°C.

‡ The mean titers of volunteers in each group are expressed in net OD units.

§ Symptoms include loose stools, abdominal cramps, anorexia, vomiting, malaise, and headache.

‖ Numbers in brackets represent geometric mean peak CFU of *V. cholerae* per gram of stool in those volunteers with positive stool cultures.

¶ Duodenal string cultures.

** Statistical comparisons (student's *t* test): a + b vs. c + d, $p < 0.0001$; e + f vs. g + h, $p < 0.001$.

ponents of the vibrio surface in attachment and colonization including LPS (11), flagellar sheath and other flagellar proteins (12), a 25-kD outer membrane protein named *ompV* (13, 14), as well as other outer membrane proteins (15) and various hemagglutinins (16). Taylor et al. recently demonstrated that expression of TcpA affected the colonizing capacity of classical *V. cholerae* 395 in a mouse model (3). However, cholera is an infection limited to the human host, and results from animal models ultimately must be confirmed in man. The studies reported herein are the first uncontrovertible demonstration that a specific vibrio surface structure is essential for colonization of the human small intestine. Genetic sequences specific for *tcpA* have been found in all pathogenic *V. cholerae* O1 isolates examined (6), suggesting that toxin-coregulated pili may represent a common mechanism for attachment and colonization. Since protection against clinical cholera developed only in those volunteers who were immunized with strain 395-N1 and not in those ingesting the noncolonizing derivative TCP2, it is clear that live attenuated, orally administered *V. cholerae* O1 vaccines should express critical colonization factors such as TcpA. The incorporation of *TcpA* and other ToxR-regulated gene products (3, 6) into killed *V. cholerae* vaccines may have advantages as well. An important future area of investigation will be the intensive study of the human mucosal response to this colonization factor.

These studies have also shown for the first time that a site-directed mutation in a regulatory gene (*toxR*) that controls the expression of virulence factors (3) can drastically reduce the colonization of the human host by a bacterial pathogen. This result indicates that the regulation of virulence gene expression can play a role as essential as that played by the structural virulence genes themselves. The importance of coordinate regulatory systems in bacterial pathogenesis is thus demonstrated.

Summary

Isogenic mutant strains of *V. cholerae* O1 lacking elements of a genetic regulon controlled by *toxR* and implicated in virulence were tested in volunteers. A deletion mutation in *ctxA*, the gene encoding the A subunit of cholera toxin, markedly attenuated disease symptoms without affecting intestinal colonization. Deletion of *toxR*, the gene encoding the cholera toxin-positive regulatory protein resulted in a diminution in colonizing capacity. A deletion mutation in *tcpA*, encoding the major subunit of the toxin coregulated pilus (regulated by *toxR*), abolished the colonizing capacity

TABLE III

Protection against Clinical Cholera Due to Classical Ogawa 395 Conferred by Prior Administration of Attenuated V. cholerae Strains 395-N1 and TCP2

Group description	No. of volunteers with diarrhea	Mean diarrheal stool volume*	Mean number of diarrheal stools*
		ml	
Controls	6/7	5,385	15
TCP2 recipients‡	2/3	2,954	10
395-N1 recipients‡	1/4	286	3

* Values for 395-N1 recipients lay outside the 95% confidence limits of the control group (data log transformed).

‡ Approximately 4 wk earlier, these individuals ingested a single dose of 10^6 viable organisms with $NaHCO_3$.

of this strain. These results show for the first time the role of a specific pilus structure in colonization of the human intestine by *V. cholerae* O1 and exemplify the significance of a genetic regulon in pathogenesis.

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