The OmpU Outer Membrane Protein, a Potential Adherence Factor of *Vibrio cholerae*

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Expression of the OmpU outer membrane protein of *Vibrio cholerae* is positively regulated by *toxR*, which also regulates critical virulence factors such as cholera toxin and the toxin-coregulated pilus colonization factor. In this study, we have characterized the 38-kDa OmpU protein and investigated its role in the adhesion of *V. cholerae* to mammalian cells. The amino-terminal sequence of OmpU has similarity with the sequences of *Haemophilus influenzae* HMW1 and HMW2 adhesins, which, in turn, also have similarity with the sequence of *Bordetella pertussis* filamentous hemagglutinin. A monoclonal antibody directed against FHA recognized both *V. cholerae* OmpU and *Escherichia coli* OmpA, and polyclonal anti-OmpU antibodies recognized FHA and *E. coli* OmpA, suggesting the existence of common epitopes among these proteins. OmpU was strongly recognized by convalescent-phase serum from volunteers experimentally infected with virulent *V. cholerae* strains, indicating that OmpU is immunogenic and produced in vivo. OmpU selectively bound to fibronectin and to an arginine-glycine-asparagine (RGD) tripeptide but not to other matrix glycoproteins tested such as collagen or laminin. Antibodies directed against OmpU or their F(ab)₂ fragments completely inhibited adhesion of several *V. cholerae* strains to HeLa, HEP-2, Caco-2, and Henle 407 epithelial cells and also inhibited intestinal colonization and conferred protection in newborn mice against both biotypes (El Tor and classical) of *V. cholerae* O1. Collectively, these data indicate that OmpU has adhesive properties which may play a role in the pathogenesis of cholera.

Vibrio cholerae causes cholera by colonizing the small intestine and elaborating a potent enterotoxin, cholera toxin (CT). Although the toxin responsible for the severe dehydrating diarrhea is well characterized, the adhesins responsible for small-bowel colonization are not fully characterized. Several potential adherence factors have been described for *V. cholerae*, including surface proteins, hemagglutinins, and several types of pili (7, 9, 13, 38–40). The toxin-coregulated pilus (TCP) is essential for intestinal colonization by classical biotype strain 395 in mice and humans (10, 39), and both TCP (32) and a mannose-sensitive hemagglutinin (MSHA) have been proposed as essential colonization factors in El Tor biotype strains (28). Intestinal colonization by *V. cholerae* appears to be a complex process, possibly involving sequential or simultaneous expression of several factors.

Toxin production in *V. cholerae* is part of a regulated response in which several other virulence factors in addition to CT are expressed under the control of a central regulator, ToxR (6). At least 17 ToxR-activated genes have been described (29), including genes encoding CT (24), the TCP colonization factor (39), an accessory colonization factor (29), and the outer membrane proteins (OMPs) OmpU and OmpT (25). Expression of CT, TcpA, OmpT, and OmpU is affected by changes in osmolarity and amino acid concentrations, while other environmental signals, such as temperature and pH, have a more pronounced effect on the expression of CT and TcpA

than on the OMPs (25). The 38-kDa OmpU and the 40-kDa OmpT are regulated in opposing fashions such that expression of OmpU is positively regulated by ToxR and expression of OmpT is negatively regulated.

While some genes of the *toxR* regulon such as *ctx* and *tcpA* have been cloned and their protein products have been extensively characterized, other ToxR-regulated factors such as OmpU have not been studied in detail. In this report, we describe the biochemical, immunological, and functional characterization of OmpU and present data showing that this protein may be an adherence factor of *V. cholerae*.

MATERIALS AND METHODS

Strains and plasmids. The strains used in this study include *V. cholerae* 395 (O1, classical Inaba) (20), *V. cholerae* E7946 (O1, El Tor Ogawa) (20), *V. cholerae* 569B (O1, classical Inaba), *V. cholerae* CVD101 ($\Delta ctxA$, derived from 395) (15), *V. cholerae* CVD110 ($\Delta ctxA \Delta zot \Delta ace \Delta cep \Delta orfU hlyA::mer-ctxB$; derived from E7946) (22), *V. cholerae* CVD103-HgR ($\Delta ctxA hlyA::mer$; derived from 569B) (17, 21), *V. cholerae* TCP2 ($\Delta tcpA$; derived from 395) (37), *V. cholerae* AI1837 (serogroup O139) (26), and *V. cholerae* 1A and E6 (nonencapsulated mutants of AI1837) (5). E7946Sm^r and 395Sm^r are spontaneous streptomycin-resistant mutants of E7946 and 395, respectively. *V. cholerae* CVD639 (4), a *toxR* mutant of *V. cholerae* 395, was constructed essentially as described by Miller and Mekalanos (25). Specifically, a 630-bp *EcoRI-HpaI* fragment consisting of internal *toxR* sequences was cloned into the suicide vector pGP704 to yield the plasmid pCVD 850. This plasmid was mobilized into strain 395 with selection for the Ap^r resistance marker on the vector. Integration of the plasmid into the chromosome via homologous recombination results in disruption of the *toxR* gene. The construction of CVD639 (*toxR*::pCVD 850) was confirmed by Southern hybridization. The *Escherichia coli* strains used in this study included *E. coli* DH5 α [F[–] ϕ 80dlacZ Δ M15(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(r_K[–] m_K⁺) *deoR supE44 thi-1 gyrA96 relA1 λ [–]*], *E. coli* PAP702 (OmpA[–]) (a gift from Anthony Pugsley, Pasteur Institut, Paris, France), and enteropathogenic *E. coli* E2348/69 (19).

OMP extraction. Bacteria were grown overnight in LB broth (pH 7.2) at 37°C

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and lysed in a French press (15,000 lb/in²), and the OMPs were separated by differential centrifugation as described by Miller and Mekalanos (25). Profiles of OMPs were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the OmpU protein was identified as a 38-kDa band whose expression was diminished in a ToxR mutant strain as originally described by these authors. The 38-kDa band was excised from the gel and used to obtain specific rabbit anti-OmpU antiserum.

Amino-terminal amino acid sequence of OmpU. For sequencing of the amino terminus of OmpU, OMPs from *V. cholerae* CVD101 were electrophoresed in an SDS-12.5% polyacrylamide gel, blotted onto Immobilon-P transfer membranes (Millipore), and stained with amido black (33). The 38-kDa (OmpU) band was excised and subjected to amino-terminal sequence determination with an Applied Biosystems automated sequencer. This analysis was kindly performed by Robert Hall at the Food and Drug Administration, Washington, D.C.

Preparation of polyclonal anti-OmpU antiserum and F(ab)₂ fragments. Polyclonal anti-OmpU antiserum was raised in rabbits by immunization with OmpU protein purified from strain CVD101 by electroelution from an SDS-12.5% polyacrylamide gel. After electroelution, the excised gel slices containing the Coomassie blue-stained 38-kDa band were homogenized and mixed with complete Freund's adjuvant and injected subcutaneously into the back of a New Zealand White rabbit. Additional doses, for a total of three, were given weekly in incomplete Freund's adjuvant. Reactivity and specificity of the antiserum against the 38-kDa protein were assessed by immunoblotting. The antiserum was used without further absorption. F(ab)₂ fragments were obtained by digesting the anti-OmpU antibodies with pepsin, followed by purification with the Immopure F(ab)₂ preparation kit (Pierce, Rockford, Ill.).

SDS-polyacrylamide gels and Western blots (immunoblots). OMP preparations from *V. cholerae* and *E. coli* strains were denatured at 100°C, electrophoresed in SDS-12.5% polyacrylamide gels, and reacted in immunoblots with anti-OmpU antiserum (1:500 dilution), anti-filamentous hemagglutinin (FHA) monoclonal antibody X3C (1:500) (a gift from Michael Brennan [18]), and sera from human volunteers (1:100) previously infected with *V. cholerae* 395 and E7946 (20). For the human sera, the membrane containing the OMPs was blocked with 0.5% Tween 20-3% milk in phosphate-buffered saline (PBS) for 2 h at room temperature (RT). After washing, the human serum was added at a dilution of 1:100 in 0.5% Tween 20-PBS and incubated for 1 h. After washing, appropriate human or rabbit secondary antibodies (Sigma) were added and detected with the alkaline phosphatase substrate (Promega).

Adhesion of OMPs to immobilized matrix proteins and RGD. Adhesion of OMPs to immobilized matrix proteins (fibronectin, laminin, and collagen) and the arginine-glycine-aspartic acid (RGD) tripeptide was studied by an enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with fibronectin, collagen, laminin (Sigma), or RGD (synthesized at the Biopolymer Laboratory, University of Maryland) at a concentration of 1 µg/ml in carbonate buffer (pH 9.5) overnight at 4°C, washed three times with PBS buffer-0.5% Tween 20, blocked with PBS-0.5% Tween 20-1% bovine serum albumin (BSA) for 2 h at RT, and washed again three more times. OMP preparations from *V. cholerae* CVD101 (prepared as described above), diluted 1:100 in PBS-0.5% Tween 20, were added for 1 h at RT, washed three times, and incubated for 1 h at RT with anti-OmpU serum diluted 1:100 in PBS-0.5% Tween 20. The plates were then washed, incubated for 1 h at RT with rabbit immunoglobulin G alkaline phosphatase conjugate (Sigma) diluted 1:1,000 in PBS-0.5% Tween 20, washed again, and incubated with alkaline phosphatase substrate for 30 min, and the *A*₄₀₅ was read.

Inhibition of binding of OmpU to fibronectin by RGD was assayed by ELISA, with one step added to the procedure described above. After blocking with PBS-0.5% Tween 20-1% BSA for 2 h at RT, the plates were washed and incubated for 1 h with RGD (1:10, 1:25, 1:50, 1:100, 1:500, and 1:1,000), and then the reaction was continued as described above, adding the OMPs and so on. The same procedure was used in an attempt to inhibit binding of OmpU to RGD by fibronectin.

Adhesion of *V. cholerae* to cultured cells. Adhesion assays of *V. cholerae* 395, E7946, 569B, CVD103-HgR, and TCP2, and enteropathogenic *E. coli* E2348/69 to HEp-2, HeLa, and Henle 407 cell monolayers were performed in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 1% D-mannose. An inoculum of 10⁷ bacteria (grown in LB broth overnight at 37°C [pH 7.2], unless stated otherwise) was added to the cell monolayers for 3 h at 37°C in a 5% CO₂ atmosphere. The cells were washed three times with PBS, fixed with 100% methanol at RT for 20 min, stained with Giemsa for 20 min at RT, and visualized by light microscopy. For quantitative assays, 10⁷ CFU of bacteria grown statically overnight in LB broth were added to cell monolayers in a 24-well tissue culture plate. After 30 min of incubation (37°C, 5% CO₂), the monolayers were washed three times with PBS. Two hundred microliters of 1% Triton X-100 (in PBS) was added to each well, and the plate was rocked at RT for 10 to 15 min. Eight hundred microliters of LB broth was then added and vigorously pipetted up and down to thoroughly mix the bacteria and lysed HEp-2 cells. The suspensions were serially diluted and plated on L agar to determine the number of bacteria that were cell associated. Adherence is expressed as the percentage of the inoculum which was recovered from the HEp-2 cells after 30 min of incubation. For assays in which various dilutions of anti-OmpU and anti-TCP antisera were added to test for inhibition of HEp-2 cell adherence,

FIG. 1. OMP profiles (A) and Western blot analysis with anti-OmpU serum (B) of *V. cholerae* and *E. coli* strains separated by SDS-12.5% PAGE. Positions of the 38-kDa OmpU and 40-kDa OmpT are indicated. Lanes: A and J, molecular mass markers; B, *V. cholerae* CVD101; C, *V. cholerae* CVD639 (ToxR⁻); D, *V. cholerae* CVD110; E, *V. cholerae* 569B; F, *V. cholerae* CVD103-HgR; G, *V. cholerae* AI1837; H, *E. coli* DH5α; I, *E. coli* PAP702 (OmpA⁻).

strains were grown in LB broth [pH 6.5] at 30°C overnight, which are optimal conditions for expression of TCP by classical *V. cholerae* strains.

Inhibition of bacterial adhesion to HEp-2 cells with specific antibodies was performed by preincubating the cells and the bacteria for 20 min at RT with the anti-OmpU serum, the F(ab)₂ fragments of anti-OmpU serum, or anti-TCP serum at the indicated dilutions; the adhesion assay described above followed. The anti-TCP serum was prepared against TCP purified from *V. cholerae* O1 strain 395 (9).

Animal model for passive protection. The infant mouse animal model (2, 8) was used to determine the protective potential of the anti-OmpU serum and anti-OmpU F(ab)₂ fragments in vivo. Bacteria (*V. cholerae* 395Sm^r and E7946Sm^r) were grown overnight at 37°C, centrifuged at 8,000 × g for 10 min at 4°C, and washed with PBS buffer, and the concentration was adjusted to 10¹⁰ vibrios per ml. The bacteria were centrifuged again at 13,000 × g for 5 min, after which the pellet was resuspended in the same volume with the antiserum preparation (1:10 in PBS) and incubated for 1 h at RT. Infant mice (3 to 5 days old) were inoculated orally with 50 µl of the vibrio-antiserum mixture (5 × 10⁸ vibrios). Protection was determined by mouse survival after 48 h for strain 395 and 24 h for strain E7946.

RESULTS

Isolation and characterization of OmpU. OmpU is a major OMP of *V. cholerae* with an apparent molecular mass of 38 kDa in SDS-polyacrylamide gels (Fig. 1A). The identity of this 38-kDa protein as OmpU was confirmed by its diminished expression in a ToxR mutant strain (Fig. 1A, lane C) as originally described by Miller and Mekalanos (25). Polyclonal antiserum raised against purified OmpU recognized only the 38-kDa polypeptide in the outer membrane fractions of all of the *V. cholerae* strains examined (Fig. 1B). On the basis of this specificity, we attempted to clone the gene encoding OmpU by

FIG. 2. (A) Similarity among amino acid sequences of OmpU amino terminus and *H. influenzae* HMW1 and HMW2 adhesins (numbers indicate amino acid residue numbers for HMW1 and HMW2); (B) Western blot analysis with the FHA monoclonal antibody X3C. Lanes: A and E, molecular mass markers; B, OMPs from *V. cholerae* CVD101; C, OMPs from *E. coli* DH5 α ; D, whole-cell lysate of enteropathogenic *E. coli* E2348/69.

screening a genomic expression library of *V. cholerae* 395 in *E. coli* DH5 α with the anti-OmpU serum. However, this library showed high background reactivity with the anti-OmpU serum, making it difficult to screen putative clones. When this antiserum was absorbed with the host *E. coli* strain, all reactivity to the OmpU protein was lost (data not shown). We subsequently found that the high background was due to cross-reactivity between OmpU and the OmpA protein of *E. coli*. Anti-OmpU antibodies recognized a 34-kDa protein in *E. coli* DH5 α (Fig. 1B, lane H) that was not present in an OmpA *E. coli* mutant (Fig. 1B, lane I). OmpA is a heat-modifiable, major OMP of *E. coli* which has an apparent molecular mass of 28 kDa in SDS-polyacrylamide gels if extracted at room temperature but which runs as a 33.5-kDa polypeptide when completely denatured at 100°C (3). Similarly, *V. cholerae* OmpU runs as a 38-kDa polypeptide after heating at 100°C but as a 32-kDa protein when heated at 50°C (data not shown).

Amino-terminal sequence similarities and cross-reactivities. The amino-terminal amino acid sequence was determined, and a search of the GenBank and EMBL sequence databases revealed an interesting similarity between this sequence and the sequences of *Haemophilus influenzae* HMW1 and HMW2 adhesins (Fig. 2A). These proteins mediate attachment of non-typeable *H. influenzae* to human epithelial cells (35) and also share similarity with the *Bordetella pertussis* FHA (1). Although the amino-terminal sequence of OmpU showed little similarity to that of FHA, a monoclonal antibody (X3C) against FHA (18) recognized both *V. cholerae* OmpU and *E. coli* OmpA in Western blots (Fig. 2B), and anti-OmpU antibodies also recognized FHA (data not shown). The exact epitope recognized

by this monoclonal antibody is not known, but these results suggest that OmpU and FHA share epitopes in a region other than the amino-terminal region of OmpU.

Binding to fibronectin. A number of bacterial adhesive factors such as *E. coli* curli (27) bind to fibronectin and other matrix glycoproteins. For *B. pertussis* FHA, binding to eukaryotic cells involves interaction with an RGD tripeptide (31). We examined the ability of OmpU to bind to matrix glycoproteins, namely, fibronectin, collagens, and laminin, and to an RGD tripeptide by ELISA. OmpU selectively bound to immobilized fibronectin and to the tripeptide RGD but did not bind to laminin or collagen (Fig. 3). In competitive binding studies, fibronectin did not inhibit binding of OmpU to RGD, although it did inhibit binding of OmpU to fibronectin (data not shown). Similarly, RGD did not inhibit binding of OmpU to fibronectin, suggesting that the binding of OmpU to fibronectin and RGD involves different regions of the protein. Comparison of outer membrane samples prepared from the ToxR-negative strain CVD639 (which produces lower levels of OmpU than strains 395 and CVD101 do) and the ToxR-positive strain CVD101 showed lower binding for proteins from CVD639 (Table 1).

Role of OmpU in adherence to epithelial cells. The role of OmpU in adherence of *V. cholerae* to cultured epithelial cells was examined by light microscopy and Giemsa staining. In preliminary experiments, *V. cholerae* 395 and E7946 were observed to adhere to HEp-2, HeLa, Caco-2, and Henle 407 cells (data not shown). Since the patterns of adherence were the same in all cell lines, we selected HEp-2 cells for further experiments. Most *V. cholerae* strains adhered avidly to HEp-2 cells, and in quantitative counts, 15.7% of the initial inoculum of *V. cholerae* CVD101 and 20% of the initial inoculum of CVD110 adhered to HEp-2 cells after 30 min of incubation. Strains 569B and CVD103-HgR adhered, but at much lower levels, i.e., 1.1 and 0.6%, respectively. ToxR mutant CVD639 adhered at 1.1% of the level of the initial inoculum. To test whether this adherence was mediated by OmpU, anti-OmpU serum was added at dilutions of 1:10 through 1:1,000 (Table 2). For all strains tested, adherence to HEp-2 cells was greatly reduced at higher concentrations of anti-OmpU serum but was restored to normal levels as the serum was diluted. In contrast, addition of antisera prepared against the TCP did not inhibit adherence of these strains (Table 2). We examined additional

FIG. 3. Adhesion of OmpU to immobilized matrix proteins and the RGD tripeptide. Assays were performed in triplicate. Negative controls included wells without anti-OmpU serum and wells without immunoglobulin G-alkaline phosphatase conjugate (No Ig-AP).

TABLE 1. Binding of OMPs from *V. cholerae* CVD101 (ToxR⁺) and CVD639 (ToxR⁻) to fibronectin (FN) or RGD tripeptide

Substrate	Dilution	<i>A</i> ₄₀₅ ^a		
		OMPs from CVD101	OMPs from CVD639	Pure OmpU
FN	1:500	1.135	0.819	1.270
	1:1,000	1.525	1.029	1.486
	1:2,000	1.531	1.363	1.544
RGD	1:50	1.739	1.688	1.806
	1:500	1.731	1.552	1.823
	1:1,000	1.607	1.564	1.553

^a Results are means of three readings.

strains with 1:10 dilutions of anti-OmpU antisera and examined them microscopically, by use of a scoring system of + to indicate adherence by >10 bacteria per microscopic field and – for 0 to 2 adherent bacteria per field (Table 3). As expected, the negative control of enteropathogenic *E. coli* E2348/69 adhered in a fashion that was not inhibitable by antisera against OmpU or TCP. All *V. cholerae* strains tested adhered to HEp-2 cells, and this adherence was not inhibited by anti-TCP sera. With one exception, adherence of the *V. cholerae* strains was inhibited by the addition of either whole anti-OmpU serum or by purified F(ab)₂ fragments of anti-OmpU antiserum at concentrations of 8, 3.2, and 1.6 mg/ml. The one exception was an O139 strain, AI1837, whose adherence was not inhibited by anti-OmpU serum. Because this strain possesses a polysaccharide capsule, we examined two nonencapsulated mutants of AI1837 for HEp-2 adherence. These mutants, 1A and 6E, still adhered to HEp-2 cells at levels comparable to those of O1 strains, but in contrast to the parent O139 strain, adherence was completely inhibited by anti-OmpU serum (Table 3). These results suggest that the capsule of O139 acts as a masking component, impeding recognition of OmpU by antibodies.

Passive protection of newborn mice with anti-OmpU serum.

Both anti-OmpU serum and purified F(ab)₂ fragments conferred protection against infection with *V. cholerae* 395 (classical biotype) or E7946 (El Tor biotype) in newborn mice (Table 4). When *V. cholerae* 395 was inoculated together with rabbit preimmune serum, none of four newborn mice survived after 48 h (Table 4). As a positive control, strain 395 was inoculated together with anti-TCP serum, which was previously shown to protect infant mice from challenge (36). In this group, three of four mice survived the challenge. In the group

TABLE 2. Adherence of *V. cholerae* to HEp-2 cells in the presence of anti-OmpU or anti-TCP sera

Antiserum and dilution	% Recovery of initial inoculum ^a in strain:			
	395	E7946	569B	CVD103-HgR
No antiserum	37.9	50.5	0.4	1.9
With anti-OmpU				
1:10	0.2	5.7	<0.1	0.1
1:50	10.0	5.8	0.1	0.1
1:100	20.9	18.5	0.2	0.3
1:500	27.3	21.4	0.4	1.9
1:1,000	36.8	48.7	0.4	1.9
With anti-TCP				
1:10	36.7	49.3	0.4	1.9
1:50	37.8	49.4	0.4	1.9
1:100	37.7	50.5	0.4	1.9
1:500	37.7	50.3	0.4	1.9
1:1,000	38.0	50.1	0.4	1.9

^a Initial inoculum was 10⁷ bacteria per 10⁵ HEp-2 cells.TABLE 3. Adhesion of *V. cholerae* strains to HEp-2 cells in the presence or absence of anti-OmpU serum, F(ab)₂ fragments purified from the anti-OmpU serum, or anti-TCP serum

Strain	Adhesion to HEp-2 cells in presence of:			
	No addition	Anti-OmpU serum	Anti-OmpU F(ab) ₂ fragments	Anti-TCP serum
<i>V. cholerae</i> O1 395	+	–	–	+
<i>V. cholerae</i> O1 E7946	+	–	–	+
<i>V. cholerae</i> O1 TCP2	+	–	–	+
<i>V. cholerae</i> O1 569B	+	–	–	+
<i>V. cholerae</i> O1 CVD103-HgR	+	–	–	+
<i>V. cholerae</i> O1 CVD639	+	–	–	+
<i>V. cholerae</i> O139 AI1837	+	+	+	+
<i>V. cholerae</i> O139 1A	+	–	–	+
<i>V. cholerae</i> O139 6E	+	–	–	+
<i>E. coli</i> E2348/69	+	+	+	+

of mice inoculated with *V. cholerae* 395 together with anti-OmpU serum, five of five survived, and in the group inoculated with 395 and anti-OmpU F(ab)₂ fragments, three of four survived (Table 4). Similar results were seen in mice challenged with El Tor strain E7946 (Table 4).

Immunogenicity of convalescent-phase human sera to OmpU.

Postchallenge sera from volunteers experimentally infected with *V. cholerae* 395 or E7946 strongly recognized OmpU and OmpT in Western blots (Fig. 4), while prechallenge sera from these volunteers recognized OmpU weakly, probably as a result of cross-reactivity with OmpA of normal flora *E. coli*.

DISCUSSION

The OmpU OMP of *V. cholerae* was initially described as part of the *toxR* regulon of this species (25). Although a variety of other genes regulated by ToxR have been cloned and characterized, including genes encoding CT (24), the TCP (39), and an accessory colonization factor (29), OmpU has not heretofore been characterized at either the functional or genetic level. The fact that expression of OmpU is coregulated with expression of critical virulence factors such as CT and the TCP suggests that OmpU is also an important virulence factor for this species (25). We now present evidence that OmpU acts as an adherence factor involved in colonization of epithelial cells by *V. cholerae*.

We examined the adherence of OmpU to fibronectin, collagen, laminin, and the RGD tripeptide and found that OmpU bound to fibronectin and RGD. Although the *in vivo* relevance of these results is unknown, they indicated that OmpU could bind to components of eukaryotic cells. We then examined the role of OmpU in adherence of *V. cholerae* to cultured epithelial cells. All *V. cholerae* O1 strains tested were found to adhere to HEp-2 cells, and adherence was completely inhibited in the

TABLE 4. Protection of newborn mice against *V. cholerae* challenge with anti-OmpU serum and anti-OmpU F(ab)₂ fragments

Bacterial strain	Antiserum	No. of surviving mice/total no.
395	Preimmune serum	0/4
	Anti-OmpU serum	5/5
	Anti-OmpU F(ab) ₂ fragments	3/4
	Anti-TcpA serum	3/4
E7946	Preimmune serum	3/10
	Anti-OmpU serum	5/9
	Anti-OmpU F(ab) ₂ fragments	7/10

FIG. 4. Western blot analysis of *V. cholerae* or *E. coli* OMPs with sera from volunteers experimentally infected with *V. cholerae* 395 or E7946. Lanes: A, molecular mass markers; B, OMPs of *V. cholerae* CVD101 stained with amido black; C, prechallenge serum from volunteer fed *V. cholerae* 395; D, postchallenge serum from volunteer fed *V. cholerae* 395; E, postchallenge serum from volunteer fed *V. cholerae* E7946; F, prechallenge serum from volunteer fed *V. cholerae* E7946.

presence of polyclonal antiserum against OmpU. Purified F(ab)₂ fragments of this serum also inhibited adhesion, showing that inhibition of adherence by the antiserum was not due to non-specific inhibition by the Fc fragment of the antibodies. Adherence of *V. cholerae* to HEp-2 cells was not mediated by TCP, since a derivative of strain 395 specifically mutated in the *tcpA* gene adhered to HEp-2 cells at levels comparable with those seen with strain 395 and antiserum against TCP did not inhibit adherence of *V. cholerae* to HEp-2 cells.

A prototypic strain of the newly emerged O139 serogroup (AI1837) was also shown to adhere to HEp-2 cells (Table 2). In contrast to the results with O1 strains, adherence was not inhibited by either the anti-OmpU antiserum or its F(ab)₂ fragments. This strain, however, has a polysaccharide capsule (12) that perhaps prevents the antibodies from interacting with OmpU. When nonencapsulated mutants (1A and E6) were tested in this assay, they were observed to still adhere to HEp-2 cells, but this adherence was now inhibited by anti-OmpU serum. One possible explanation for these results is that for the O139 serogroup, both the polysaccharide capsule and OmpU can mediate adherence to cultured epithelial cells, although other possibilities cannot be ruled out. A polysaccharide capsule has been reported previously to mediate adherence of a non-O1/non-O139 *V. cholerae* strain to cultured epithelial cells (11). It has also been shown that encapsulated cells of *V. cholerae* O139 tend to agglutinate, which contributes to the adherence to human intestinal mucosa (41).

The amounts of OmpU produced by different *V. cholerae* strains were not identical. Expression of OmpU by the ToxR⁻ mutant was reduced but not totally absent, suggesting that OmpU may have a constitutive expression that is independent of ToxR. Adherence of the ToxR⁻ mutant to HEp-2 cells was also reduced compared with the isogenic parent strain. Strain

569B also produced less OmpU than did CVD101, a derivative of strain 395. The diminished expression of OmpU in this strain correlated with reduced adherence to HEp-2 cells since adherence of 569B was reduced 14-fold relative to adherence of CVD101, a derivative of 395. Strain 569B carries a chromosomal deletion of the *toxS* gene (23) which affects expression of CT, and this mutation may have an effect on expression of OmpU. This strain also shows diminished intestinal colonization in humans (21), mice (16), and rabbits (30).

CVD103-HgR produces even less of the 38-kDa OmpU protein and adheres to HEp-2 cells at low levels. CVD103-HgR is a derivative of CVD103, which in turn was derived from 569B (17). CVD103-HgR is recovered from the stools of immunized volunteers at lower levels than those of CVD103 or 569B (21), and quantitative studies in infant mice show that intestinal colonization by CVD103-HgR is diminished relative to that by CVD103 or 569B (17). A linkage of OmpU expression and in vivo or in vitro adherence, although consistent with the inhibitory effect seen with anti-OmpU serum, is only speculative until isogenic strains differing in the *ompU* locus are constructed and tested.

In addition to the in vitro studies supporting a role for OmpU as an adherence factor, in vivo evidence of such a role was sought. OmpU appears to be expressed in vivo during human infection, since postchallenge sera of volunteers fed *V. cholerae* 395 and E7946 strongly recognize OmpU (Fig. 4). However, OmpU is weakly recognized by prechallenge sera of these volunteers, which may be due to the fact that OmpU shares epitopes with OmpA of *E. coli* (Fig. 1). When tested in the infant mouse model, both whole antiserum raised against OmpU and purified F(ab)₂ fragments of this antiserum conferred protection against *V. cholerae* O1 challenge by either biotype (Table 4). Sengupta et al. (34) previously reported that antibodies directed against *V. cholerae* OMPs in the 40- to 43-kDa size range provided ca. 80% protection against challenge with *V. cholerae* O1 of either biotype in the infant mouse model. Examination of the SDS-polyacrylamide gels published by these investigators suggests that this protective fraction probably includes OmpU.

Among the potential intestinal colonization factors of *V. cholerae*, the TCP is the most firmly established. The works of Rhine and Taylor (32), Sun et al. (36), and Taylor et al. (39) have shown that mutation of *tcpA* greatly reduces intestinal colonization in infant mice and that antibodies against TCP can passively protect mice against challenge with either biotype. The importance of TCP in human infection was demonstrated when a *tcpA* mutant of classical *V. cholerae* 395 failed to colonize volunteers (10). Other workers, however, have questioned the importance of TCP in disease due to the El Tor biotype and proposed that the MSHA pili are more important in El Tor infection. Osek et al. (28) reported that antibodies directed against TCP inhibited colonization in the infant mouse model by classical but not El Tor strains, whereas antibodies against MSHA can block colonization by strains of the El Tor biotype but not by classical strains. While the reason for these discrepant results is not known, the results of Osek and colleagues suggest that there are specific colonization factors for each of the O1 biotypes. (MSHA is also expressed by the O139 serogroup [14].) Our results show that the putative OmpU adhesin is expressed in both biotypes of O1 and O139 and, at least in cultured epithelial cells, appears to play an essential role in adherence by O1 and unencapsulated O139 strains. Construction of isogenic strains that differ in one or more of these putative adhesins will allow the determination of the relative importance of TCP, MSHA, and OmpU in intestinal colonization by *V. cholerae*.

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