The Serological Properties of the Cell Surface Proteins of *Vibrio cholerae*

By SHAHJAHAN KABIR†

The National Bacteriological Laboratory, Stockholm, Sweden and the International Centre for Diarrhoeal Disease Research, Dacca, Bangladesh

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The serological properties of cell surface proteins of *Vibrio cholerae* belonging to both the biotypes (classical and El Tor) and the serotypes (Ogawa and Inaba) were investigated. Proteins were isolated by extracting *V. cholerae* with EDTA in the presence of sodium chloride. The surface localization of these proteins was confirmed with (a) radioiodinated protein A as an immunoprobe and (b) antiserum absorption studies with whole bacteria. There were similarities among the polypeptides of cell surface proteins isolated from various *V. cholerae* types. Antisera to these proteins agglutinated several *V. cholerae* strains, irrespective of biotype, serotype and antibiotic sensitivity. The antisera did not agglutinate pathogenic enteric bacteria such as enterotoxigenic *Escherichia coli*, *Salmonella typhi*, *Shigella* spp., *Aeromonas hydrophila* and *Yersinia enterocolitica*. The cell surface proteins of *V. cholerae* were immunogenic in rabbits as high titres of anti-protein specific antibodies were detected by the ELISA technique in the immune sera. These results suggest that the cell surface proteins are common antigens of *V. cholerae* and can be developed as a potential vaccine candidate against cholera.

INTRODUCTION

Vibrio cholerae is a Gram-negative bacterium which colonizes the small intestine. There it secretes a protein toxin which binds to the mucosal epithelial cells and stimulates adenylate cyclase activity in the intestinal tract. The process alters ion transport at the mucosal surface and finally causes diarrhoea (Field, 1971). Clinical cholera induces both antitoxic and antibacterial immunities in the infected host (Majumdar *et al.*, 1981). Although cholera is a toxin-mediated disease, recent volunteer studies have demonstrated that antibacterial immunity was more important in protecting human beings than antitoxic immunity (Levine *et al.*, 1979).

The surface of *V. cholerae*, like other Gram-negative bacteria, consists of macromolecules including proteins and LPS (Kabir & Mann, 1980; Kabir, 1980). Among the various cell surface components of *V. cholerae* examined, proteins have been found to be lacking toxic properties (Kabir & Mann, 1980). Diarrhoea in man may be caused by several enteric bacteria such as *V. cholerae* (both O1 and non-O1 strains), *V. parahaemolyticus*, enterotoxigenic *Escherichia coli*, *Aeromonas hydrophila*, *Shigella* spp. and *Yersinia enterocolitica* (Craig, 1980). Whether *V. cholerae* shares cross-reacting surface protein antigens with other members of enteric bacteria causing diarrhoeal illness has not been investigated. Also, little is known about the immunogenicity of cell surface proteins of *V. cholerae*.

The present investigation was initiated (i) to study the serological properties of surface proteins of *V. cholerae* belonging to both biotypes (classical and El Tor) and both serotypes (Ogawa and Inaba), (ii) to investigate the antigenic cross-reactivity of cell surface proteins of *V. cholerae* to other members of enteric bacteria causing diarrhoeal illness, and (iii) to determine the immune response to cell surface proteins of *V. cholerae* in animals such as rabbits.

† Address for correspondence: Tobakspinnargatan 5, 117 36 Stockholm, Sweden.

METHODS

Bacterial strains. The majority of cultures were obtained from the collection of the International Centre for Diarrhoeal Disease Research, Bangladesh (Table 1). A total of 117 cultures of *Vibrio cholerae* O1, isolated mostly from patients suffering from cholera epidemics in Bangladesh, were used. Of these, 43 isolates were resistant to antibiotics ampicillin, kanamycin, tetracycline, streptomycin and trimethoprim-sulphamethoxazole. Nineteen enterotoxigenic *Escherichia coli* strains isolated from diarrhoeal stools were studied. Of these, six produced only the heat-labile toxin (LT), seven only the heat-stable toxin (ST) and the remaining six produced both toxins. Twelve strains of *V. cholerae* non-O1 and six strains of *Vibrio parahaemolyticus* were also included in the study. Sixteen cultures of *Shigella* spp., belonging to major serotypes (*Sh. dysenteriae*, *Sh. flexneri*, *Sh. boydii* and *Sh. sonnei*) were investigated. Also included in the study were ten cultures of *Salmonella typhi*, three cultures of *Aeromonas hydrophila* and two cultures of *Yersinia enterocolitica*.

Growth conditions. *Vibrio cholerae* cultures were grown both on solid and in liquid media. *Vibrio cholerae* cultures were plated on to solid nutrient agar (1%, w/v, Difco) and incubated at 37 °C overnight. For mass cultivation, a 3% (w/v) solution of peptone-water was used as the liquid growth medium. Bacteria were grown with continuous shaking at 37 °C and were harvested at the stationary phase of the growth (12 h). *Vibrio parahaemolyticus* was cultivated in the peptone-water containing 3% (w/v) NaCl.

Cultures of *Shigella* spp., enterotoxigenic *E. coli*, *S. typhi*, *A. hydrophila* and *Y. enterocolitica* were grown on blood agar plates at 37 °C.

Extraction of cell surface proteins of *Vibrio cholerae*. A 10 g (wet weight) sample of *V. cholerae* 395 (Ogawa) was washed twice with cold (4 °C) 0.1 M-NaCl and subsequently with cold EDTA/NaCl (25 ml, 0.12 M-EDTA/0.77 M-NaCl, pH 7.2). The combined extracts were centrifuged (20000 g, 30 min) and concentrated by negative pressure dialysis. EDTA was removed by dialysis against 0.01 M-phosphate buffered saline (PBS), pH 7.2. The dialysate was centrifuged at 10000 g for 15 min and the resulting supernate was used for further investigation.

Isolation of LPS. The LPS from *V. cholerae* 395 (Ogawa) was prepared by the phenol/water procedure as described by Westphal *et al.* (1952). The crude LPS was further purified to remove proteins and nucleic acids by procedures involving repeated ultracentrifugation at 105000 g (Westphal & Jann, 1965). Protein content was determined by the Lowry method, using bovine serum albumin as the standard.

PAGE. PAGE in the presence of SDS was performed according to the procedure of King & Laemmli (1971). Briefly, the gel (10%, w/v, acrylamide) was cast between glass plates (15 × 15 cm) to a height of 10 cm, using spacers 1.5 cm thick. A stacking gel (3%, w/v, acrylamide) 2 cm high was applied on top of the analytical gel. Electrophoresis was performed at 30 mA until the tracking dye reached the end of the analytical gel. Bovine serum albumin (68 kDa), ovalbumin (45 kDa) and cytochrome *c* (11.7 kDa) were used as marker proteins.

Preparation of antisera. Antisera against whole cells were raised in rabbits by injecting 10⁹ *V. cholerae* suspended in Freund's complete adjuvant (Difco) intramuscularly on days 0, 14, 28 and 42. Blood was collected 1 week after the last immunization.

Antisera against cell surface proteins were prepared with injection mixtures containing 1 mg protein in Freund's complete adjuvant. Rabbits were immunized as described above. Antisera were stored at -70 °C until use.

Absorption of serum with LPS. To remove immunoglobulins against the Ogawa LPS, the antisera (1 ml) was treated with the LPS (5 mg) from *V. cholerae* 395 (Ogawa) overnight at 4 °C. The LPS absorbed antisera was centrifuged at 105000 g for 4 h. The supernate was stored at -18 °C.

Localization of surface proteins of *V. cholerae* by a radioimmunochemical technique. (a) *Radioiodination of protein A.* (The protein was kindly prepared by Ms G. H. Vos-Scheperkeuter.) Protein A (Pharmacia) was labelled with ¹²⁵I by the chloramine T method (Hunter & Greenwood, 1962). Briefly, to 100 µg protein A (10 mg ml⁻¹ in 0.625 M-phosphate buffer, pH 7.5) 50 µg chloramine T (5 mg ml⁻¹ in phosphate buffer) and 0.5 mCi (18.5 MBq) carrier-free Na¹²⁵I (Amersham) were added. The mixture was incubated for 3 min at room temperature, the reaction was stopped by adding 200 µg sodium metabisulphite (10 mg Na₂S₂O₅ ml⁻¹ in phosphate buffered saline) and the sample was diluted with 400 µl PBS containing 1 mg bovine serum albumin ml⁻¹. The radioiodinated protein A was separated from ¹²⁵I by chromatography on a Sephadex G-50 column. Protein A, labelled to a specific activity of 2 µCi µg⁻¹, was eluted in the void volume.

(b) *Immunoassay with ¹²⁵I-labelled protein A.* The antisera to surface proteins of *V. cholerae* 395 was absorbed with LPS, as described above. The absorbed sera had an IgG concentration of 15 mg ml⁻¹ as determined spectrophotometrically at 280 nm by the procedure of Crumpton & Wilkinson (1963).

Cell suspensions of *V. cholerae* 395 (2 × 10⁸, 100 µl) were taken up in Eppendorf tubes and were incubated with increasing amounts of antisera (10–100 µl) at room temperature for 30 min. Cells were washed three times with phosphate-buffered saline and resuspended in a total volume of 200 µl PBS. To prevent non-specific clumping of cells, a drop of 5% (w/v) bactotryptone (Difco) was added to the cell suspension. To determine cell-bound antigen-antibody complexes, 100 µl ¹²⁵I-protein A (2.27 × 10⁶ d.p.m.) was added to the cell suspension. After incubation for 1 h, cells were centrifuged at 10000 g and the pellet was washed as described above. To check the specificity of

Table 1. *Bacterial strains used in the study*

Organism	No. of strains	Strain designation	Characteristics*	Source†
<i>Vibrio cholerae</i> O1	61	W-13021, W-28310, W-28422, W-28431, W-28432, W-28433, W-28437, W-28549, W-28586, W-28593, W-28595, W-28613, W-28616, W-28619, W-28620, X-32, X-76, X-95 U-700, U-1164, U-1194, U-1881, U-2127, U-2128, U-2352, U-2355, U-2371, U-2374, U-2568, U-2983, U-2986, U-14361, U-14490, U-14553, U-14690, U-14754, U-14805, U-17411, U-14898, W-15285, W-15746, W-16242, W-16243, W-16245, W-16355, W-16356, W-16358, W-16359, W-18230, W-18271, W-18450, W-18465, W-18468, W-18493, W-18509, W-18522, W-18556, W-18589, W-18591, T-20464, U-14365	Ogawa, El Tor Ogawa, El Tor (resistant to antibiotics ampicillin, tetracycline, streptomycin, kanamycin, and trimethoprim-sulphamethoxazole)	ICDDR,B ICDDR,B
<i>Vibrio cholerae</i> O1	46	W-28419, W-28427, W-28428, W-28430, W-28438, W-28442, W-28449, W-28451, W-28453, W-28466, W-28590, W-28591, W-28592, W-28594, W-28596, W-28597, W-28598, W-28599, W-28600, W-28624, X-17, X-18, X-19, X-22, X-23, X-36, X-69, X-116, X-122, X-123, X-131, X-180, X-232, X-235, X-370, X-376, X-378, X-453, X-454, X-501, X-503, X-504, X-508, X-513, X-540, T-19479	Inaba, El Tor	ICDDR,B
<i>Vibrio cholerae</i> O1	5	395 T-19765, T-19766, T-19767, T-19768	Ogawa, classical	India ^a ICDDR,B
<i>Vibrio cholerae</i> O1	5	569B H-22861, H-23448, H-23055, 10732	Inaba, classical	India ^b ICDDR,B NCTC ICDDR,B
<i>Vibrio cholerae</i> non-O1	12	U-3902, U-4026, U-4551, U-8281, U-10119, U-12028, U-12031, U-21051, W-2484, W-4556, W-5963, W-12379		ICDDR,B
<i>Vibrio parahaemolyticus</i>	6	U-24576, U-25077, U-25301, W-2365, W-4847, W-9078		ICDDR,B
Enterotoxigenic <i>E. coli</i>	6	W-29665, W-29891, W-30750, W-31453, Tc-218425, Tc-225725	LT ⁺	
	6	W-29350, W-29663, W-29668, W-30185, W-31286, Tc-201925	LT ⁺ , ST ⁺	
	7	W-28266, W-28738, W-29987, W-30422, W-30746, Tc-228575, Tc-216500	ST ⁺	
<i>Salmonella typhi</i>	10	U-11293, U-11628, U-11793, W-4531, W-4750, W-5037, W-5118, W-5890, W-5982, W-6040		ICDDR,B
<i>Aeromonas hydrophila</i>	3	N-4730, N-19802, N-20701		ICDDR,B
<i>Yersinia enterocolitica</i>	2	A, B		ICDDR,B
<i>Shigella</i>				
<i>Sh. sonnei</i>	3	W-13041, W-13402, W-14113		ICDDR,B
<i>Sh. boydi</i>	3	U-2187, W-3121, W-12394		ICDDR,B
<i>Sh. dysenteriae</i>	3	U-2127, W-13114, W-13114		ICDDR,B
<i>Sh. flexneri</i>	7	W-1332, W-12870, W-13132, W-13211, W-13295, W-15920, W-19332		ICDDR,B

* LT, heat labile toxin; ST, heat stable toxin.

† ICDDR,B, International Centre for Diarrhoeal Disease Research, Bangladesh; NCTC, The National Collection of Type Cultures, London, U.K.; ^a Isolated by and obtained from R. B. Sack, the Johns Hopkins University, Baltimore, Md, U.S.A.; ^b Isolated by the late N. K. Dutta. Obtained from R. B. Sack, the Johns Hopkins University, Baltimore, Md, U.S.A.

the reaction cells of *V. cholerae* 395 were incubated with the preimmune serum and ^{125}I -protein A. The cell pellet was suspended in a total volume of 200 μl PBS. the cell-bound radioactivity was determined by counting samples in a gamma counter with tetrabutylin-loaded scintillator (Gammavial C, Koch-Light).

ELISA. Cell surface proteins were suspended in 0.1 M- NaHCO_3 (pH 9.6) at the optimal concentration of 50 μg protein ml^{-1} . Individual wells of ELISA plates (Cooke Laboratory Products, Alexandria, Va., U.S.A.) were coated with the protein suspension (100 μl) at room temperature for 16 h. The antigen was discarded and the wells were washed three times with phosphate-buffered saline, pH 7.4 containing 0.05% (v/v) Tween 20 (PBST). Rabbit antisera to *V. cholerae* 395 (Ogawa) cells were preabsorbed with Ogawa LPS. Ten-fold dilutions of the test sera in PBST were added in 100 μl portions to the antigen-coated wells and incubated at room temperature for 1 h. Control wells without the antigen were also filled with antiserum. The wells were emptied and washed three times with PBST. The wells were filled with 100 μl of a sheep anti-rabbit immunoglobulin-alkaline phosphatase conjugate (specific for rabbit immunoglobulins G, M and A) and left at room temperature for 18 h. After washing three times with PBST, 100 μl of the enzyme substrate (*p*-nitrophenylphosphate, Sigma, 2 mg ml^{-1} in water) was added and the plates were incubated at 22 °C for 60 min. The reaction was terminated by the addition of 0.1 M-NaOH and the samples were read in an ELISA colorimeter at 400 nm.

Bacterial agglutination. Bacterial cultures were adjusted to 10^{10} cells ml^{-1} in 0.2 M-phosphate-buffered saline (pH 7.2). Twofold dilutions of antisera to surface protein antigens from *V. cholerae* 395 (Ogawa) were added to an equal volume of bacteria in microtitre plates. Prior to addition, the antisera were incubated in a water bath at 56 °C for 30 min. The agglutination pattern was recorded after incubation for 2 h at room temperature.

RESULTS

Composition of the EDTA extracted material of V. cholerae

The extraction of *V. cholerae* by EDTA in the presence of sodium chloride was monitored by electron microscopy; no cell lysis was observed. The polar flagellum of *V. cholerae* also remained intact. Chemical analysis of the extracted material showed it to be predominantly protein (90%).

The EDTA extracts from four strains of *V. cholerae* belonging to both biotypes (classical and El Tor) and serotypes (Ogawa and Inaba) were analysed by PAGE in the presence of SDS (Fig. 1). The extracts from each strain were resolved into several polypeptides. There were similarities in the polypeptide composition as these preparations all contained a major band of approximate molecular weight 48000. In addition polypeptides of approximate molecular weights 66000, 28000 and 13000 were present in all the strains examined. Variations were observed in a few minor bands among these strains.

Surface localization of the EDTA extracted proteins

Bacterial agglutination technique. To determine the localization of the EDTA extracted proteins, agglutination tests were done. Antisera, raised to the EDTA extracted material from *V. cholerae* 395 (classical, Ogawa) in rabbits, were absorbed with freshly grown *V. cholerae* 395 cells and were subsequently tested for bacterial agglutinating activity. While the unabsorbed antisera agglutinated whole *V. cholerae* 395 cells, the absorbed sera failed to do so.

Radioimmunochemical technique. Protein A obtained from *Staphylococcus aureus* binds to the F_c part of IgG molecules (Sjoquist *et al.*, 1972). Hence, ^{125}I -protein A has been used to detect cell surface antigens (Langone *et al.*, 1977). The anti-LPS activity of the antisera, raised to the EDTA extracted material from *V. cholerae* 395 (Ogawa), was removed by absorption with LPS of the same serotype. Cells of *V. cholerae* 395 were incubated with the absorbed antisera and later treated with ^{125}I -protein A. When 2×10^8 *V. cholerae* 395 (Ogawa) cells were incubated with 0.75 mg surface protein specific IgG, the binding of ^{125}I -protein A reached a plateau, signifying saturation of exposed proteins. The uptake of ^{125}I -protein A by *V. cholerae* was specific as very little radioactivity was bound when cells were incubated with the preimmune serum.

ELISA

To determine the antigenicity of the cell surface proteins of *V. cholerae*, ELISA tests were done with the surface proteins as the coating antigen. Rabbits were immunized with *V. cholerae* 395 and the immune sera were absorbed with LPS. As shown in Fig. 2, high titres of the cell

Fig. 1. SDS-PAGE of surface proteins of *V. cholerae* from the following strains (A) W-13021 (El Tor, Ogawa); (B) 395 (classical, Ogawa); (C) 569B (classical, Inaba) and (D) T 19479 (El Tor, Inaba).

surface protein specific antibodies were detected in the sera suggesting that these proteins were immunogenic.

Agglutination studies

Antisera to the surface proteins from *V. cholerae* 395 were tested against 117 strains of *V. cholerae* O1; the antisera agglutinated all these strains (Table 2). Of these, 43 strains showed multiple resistance to the antibiotics ampicillin, streptomycin, kanamycin, tetracycline and trimethoprim-sulphamethoxazole. These results suggest that the surface proteins represent the common antigens of *V. cholerae*.

To determine whether the surface proteins of *V. cholerae* bear serological relationship with other diarrhoea-causing members of the genus *Vibrio*, agglutination studies were done with 12

Fig. 2. Detection of anti-cell surface protein antibodies by the microtitre ELISA technique. The assay was carried out as described in the text, using cell surface proteins from *V. cholerae* 395 (classical, Ogawa) as the coating antigen. The antisera were raised in three rabbits.

Table 2. Agglutination of various diarrhoeagenic bacteria by antisera to cell surface proteins from *Vibrio cholerae* 395 (classical, Ogawa)

Agglutination reaction	No. of strains studied	Characteristic*
Positive:		
<i>Vibrio cholerae</i> O1	61	El Tor, Ogawa
<i>Vibrio cholerae</i> O1	46	El Tor, Inaba
<i>Vibrio cholerae</i> O1	5	Classical, Ogawa
<i>Vibrio cholerae</i> O1	5	Classical, Inaba
<i>Vibrio cholerae</i> O1	43	Antibiotic resistant
Negative:		
<i>Vibrio cholerae</i> non-O1	12	
<i>Vibrio parahaemolyticus</i>	6	
Enterotoxigenic <i>E. coli</i>	6	LT ⁺
Enterotoxigenic <i>E. coli</i>	7	ST ⁺
Enterotoxigenic <i>E. coli</i>	6	LT ⁺ , ST ⁺
<i>Salmonella typhi</i>	10	
<i>Shigella</i>		
<i>Sh. dysenteriae</i>	3	
<i>Sh. flexneri</i>	7	
<i>Sh. boydi</i>	3	
<i>Sh. sonnei</i>	3	
<i>Aeromonas hydrophila</i>	3	
<i>Yersinia enterocolitica</i>	2	

* Antibiotic resistant denotes resistance to tetracycline, ampicillin, kanamycin, streptomycin and trimethoprim-sulphamethoxazole; LT, heat labile toxin, ST, heat stable toxin.

strains of *V. cholerae* non-O1 and six strains of *V. parahaemolyticus*. None of these strains were agglutinated by the antisera to the surface proteins of *V. cholerae* suggesting that the surface proteins of *V. cholerae* are antigenically dissimilar to those of other pathogenic members of the genus *Vibrio* (Table 2). Further, the antisera did not agglutinate any strain from other diarrhoea causing bacteria such as enterotoxigenic *E. coli*, *S. typhi*, *A. hydrophila*, *Shigella* spp. and *Y. enterocolitica*.

DISCUSSION

Serological and antigenic properties of cell surface proteins of *V. cholerae* have been studied with proteins extracted by treatment with EDTA/NaCl. The following evidence suggests this method of extraction is specific. Firstly, by applying the radioimmunochemical technique involving the binding of ^{125}I -protein A to cell-bound immunoglobulins, it has been demonstrated in this investigation that the EDTA extracted proteins were localized on the bacterial surface. Secondly, with the ELISA test high titres of protein specific antibodies were detected in the immune rabbit sera, raised against whole bacteria. This suggests that these protein moieties, which were immunogenic, were exposed on the cell surface. Thirdly, antisera to the EDTA-extracted proteins agglutinated several strains of *V. cholerae* belonging to both biotypes and serotypes. Absorption of the antisera with living *V. cholerae* completely removed agglutinating antibodies. Finally, extracted proteins had polypeptides similar to those of the outer membrane of *V. cholerae* isolated from the cell envelope by Triton X-100 (Kabir, 1980). Although the present method produced a few more polypeptides a major component in both the preparations had a molecular weight of 48000. Recently, Geyer *et al.* (1979) have used EDTA/NaCl to isolate cell surface proteins from Gram-negative bacteria such as *Salmonella minnesota*.

There are several polypeptides shared by *V. cholerae* strains irrespective of the biotypes and serotypes. This was confirmed by PAGE which showed the presence of polypeptides of similar molecular weights. *Vibrio cholerae* is known to possess flagellar antigens which are shared by other members of the *Vibrio* family (Bhattacharyya, 1977). Hence antibodies to the flagellar antigens cannot differentiate *V. cholerae* from other pathogenic vibrios for man, such as non-O1 *V. cholerae* and *V. parahaemolyticus*. But as observed in this investigation, antisera to the surface protein antigens of *V. cholerae* are specific and hence, can be used for rapid screening of cholera vibrios from other diarrhoeagenic bacteria. At present, the serological identification of *V. cholerae* is done by the agglutination test with the polyvalent antiserum raised to both Ogawa and Inaba strains. Since serotype specificities are of little significance in clinical cholera, agglutination tests against the antisera to the surface proteins of *V. cholerae* should prove to be a quick diagnostic procedure for the identification of *V. cholerae*.

From the present investigation it appears that the surface proteins were immunogenic as high titre protein specific antibodies were detected in the immune sera. Previous investigations have shown that the surface proteins of *V. cholerae* did not possess toxic properties (Kabir & Mann, 1980). Therefore, these proteins can be explored as a potential non-toxic vaccine candidate against cholera.

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