Identification of some antigenically related outer-membrane proteins of strains of *Vibrio cholerae* O1 and non-O1 serovars involved in intestinal adhesion and the protective role of antibodies to them

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Summary. Outer-membrane proteins (OMPs) of *Vibrio cholerae* strains of O1 and non-O1 serovars were studied. Marked similarity was found in the OMP profiles of different *V. cholerae* O1 strains but the OMP profile of a non-O1 strain was somewhat different. Antigenic relatedness between the OMPs of different *V. cholerae* strains was established by enzyme-linked immunosorbent assay (ELISA). Immunoblotting experiments demonstrated that at least two OMPs of 36 and 25–26 Kda were immunogenic and common to strains of O1 and non-O1 serovars. Antiserum raised against the outer membrane of a *V. cholerae* strain, and rendered specific for its OMP by absorption with lipopolysaccharide, inhibited *in vitro* the intestinal adhesion of the homologous and heterologous strains of *V. cholerae* irrespective of their biotype, serotype and serovar. Furthermore, antiserum to OMPs induced passive protection against vibrio challenge in rabbit ileal loop experiments. These results suggest that the OMPs may be useful in immunoprophylaxis against cholera.

Introduction

The classic diarrhoeal disease “cholera” is caused by the enterotoxins produced by strains of *Vibrio cholerae* of serovar O1 growing within the gut (Finkelstein, 1973). *V. cholerae* strains of other serovars cause sporadic cases and localised outbreaks of gastroenteritis (Blake *et al.*, 1980). Examination of convalescent cholera patients shows that both antibacterial and antitoxic responses are stimulated (Majumdar *et al.*, 1981). Levine *et al.* (1979) has demonstrated the relative importance of antibacterial immunity in protection against cholera and, although this is believed to be mediated largely through the lipopolysaccharide (LPS) component of *V. cholerae* (Holmgren and Svennerholm, 1977; Chitnis *et al.*, 1982; Dasgupta and Ghose, 1988), Attridge and Rowley (1983) have suggested that non-LPS antigens may also be involved in protection.

Some outer-membrane proteins (OMPs) of *V. cholerae* are exposed on the cell surface (Manning and Hayness, 1984; Richardson and Parker, 1985) and may play a critical role in the process of

adhesion to, and subsequent colonisation of, the intestinal wall (Buchanan and Pearce, 1979; Richardson and Parker, 1985). If this is so, antibodies to OMPs might protect against vibrio challenge by inhibiting intestinal adherence. For antibodies to OMPs to be useful immunoprophylactically, those OMPs involved in the adhesion process would need to be antigenically common to different strains of *V. cholerae* irrespective of their biotype, serotype and, preferably, serovar as well. It is unfortunate that little information is available about these matters.

In this study we have examined the OMP profiles of some strains of *V. cholerae* of O1 and non-O1 serovars and used sensitive immunoassays to detect antigenically common OMPs. We have further investigated this antigenic relatedness in relation to anti-OMP serum-mediated inhibition of intestinal adhesion and protection against *V. cholerae* challenge.

Materials and methods

Bacterial strains

The strains of *V. cholerae* of serovar O1 used in this study were isolated originally from stool samples of

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patients with diarrhoea and comprised both classical and ElTor biotypes and Inaba and Ogawa serotypes. *V. cholerae* strains belonging to non-O1 serovars were isolated locally either from diarrhoeal stool specimens or from the environment, i.e., water, sediment and other sources (Datta-Roy *et al.*, 1986).

Preparation of outer membrane (OM)

Bacteria were grown in Brain Heart Infusion Broth (BHI; Difco) for 18 h at 37°C with constant shaking. Cells were harvested by centrifugation, washed with 10 mM HEPES buffer (Sigma), pH 7.0, and disrupted in an ultrasonic disintegrator (MSE) by 4 or 5 cycles each of 30-s duration. Both DNAase (Sigma) 100 µg/ml and RNAase (Sigma) 100 µg/ml were added to the cell sonicate and, after incubation at 37°C for 10 min, intact cells were removed by low speed centrifugation (750 *g* for 5 min). The disrupted cell supernate was removed, diluted with 2 volumes of 10 mM HEPES buffer, pH 7.0, and centrifuged at 105 000 *g* for 1 h at 4°C. The pellet containing the crude envelope fraction was resuspended in HEPES buffer, treated with an equal volume of Sarkosyl (sodium lauryl sarcosinate, 1% w/v Sigma) for 30 min at 24°C to solubilise selectively the inner membrane (Filip *et al.*, 1973) and centrifuged again at 105 000 *g* for 1 h at 4°C. The OM-containing pellet was washed with distilled water and stored at -20°C. OM preparations were negatively stained with phosphotungstic acid 2% w/v for 1 min and examined with a transmission electronmicroscope (Model 421 Philips, Holland) at 60 kV. Protein content of OM preparations was measured by the method of Markwell *et al.* (1978) with bovine serum albumin (BSA) as standard.

Preparation of lipopolysaccharides (LPS)

Lipopolysaccharides were extracted from acetone-dried cells of *V. cholerae* O1 and non-O1 strains with aqueous phenol at 68°C (Westphal and Jann, 1965). Protein remaining in LPS preparations was removed by digestion with pronase (Sigma) for 1 h at 37°C at an enzyme:substrate ratio of 1:100. Digested material was heated in a boiling water bath for 30 min, exhaustively dialysed against water and lyophilised.

Preparation of antisera

Antisera to OM preparations of strains of *V. cholerae* of both O1 and non-O1 serovars were raised in male albino rabbits. Increasing amounts (100, 200, 300 and 400 µg) of antigen emulsified in Freund's incomplete adjuvant were injected intramuscularly at 14-day intervals. Sera were collected 7 days after the last injection and heat inactivated (56°C for 30 min). Anti-LPS activity was removed from serum by incubating it overnight at 4°C with LPS 5 mg/ml and subsequent centrifugation at 105 000 *g* for 5 h at 4°C (Kabir, 1983). Efficacy of the

anti-LPS absorption procedure was confirmed either by an immunodiffusion test or by an enzyme-linked immunosorbent assay (ELISA). Antiserum raised against the OM preparation of a given strain of *V. cholerae* was absorbed with LPS preparation of the homologous strain.

Sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970) with a separating acrylamide gel of 12.5% w/v and a stacking gel of 3% w/v. Membrane preparations containing *c.* 70 µg of protein were dissolved in sample buffer, heated at 100°C for 5 min and electrophoresed at room temperature at constant voltage. After electrophoresis, gels were stained with Coomassie Brilliant Blue and destained by washing in a solution of methanol 5% v/v and acetic acid 7% v/v in water.

ELISA

The OM suspension, 100 µl (5 µg of protein) in 0.1 M bicarbonate buffer (pH 8.5), was added to each well of a micro-ELISA plate (Dynatech). The plate was incubated at 4°C overnight and the wells were washed with phosphate-buffered saline (PBS), pH 7.6, containing Tween-20 0.05% v/v. After washing, 100-µl volumes of BSA 1% w/v in PBS were added to each well to saturate all unbound sites and the plate was incubated for 2 h at room temperature. 100 µl-amounts of antiserum, serially diluted with BSA 0.5% w/v in PBS, were added to each well and the plate was incubated for 2 h at room temperature. After incubation and washing, 100-µl amounts of peroxidase-conjugated anti-rabbit IgG (Sigma) (diluted 1 in 1000) were added to each well and the plate was incubated for a further 2 h at room temperature. The plate was then washed and 100 µl of *o*-phenylenediamine (Sigma) 1 mg/ml in 0.1 M sodium citrate buffer, pH 4.5, containing H₂O₂ 30% v/v 2 µl/ml, was added to each well. After 15–20 min, the absorbance of the wells was measured at 450 nm in a micro-ELISA reader (Bio-Rad). Reactivity of the anti-OM sera to LPS preparations was also measured as above but with plates coated with LPS 10 µg/well.

Immunoblotting

The method of Towbin *et al.* (1979), modified by replacing fetal calf serum with Tween-20 1% v/v and BSA 3% w/v, was used. OM preparations electrophoresed on SDS-PAGE (12.5%) were transferred to nitrocellulose (pore size 0.45 µm; Millipore) at 4°C by applying a voltage of 25 V for 4 h. Blots were incubated for 2 h at 25°C with appropriate antiserum at a dilution of 1 in 100 and then incubated with peroxidase-labelled anti-rabbit IgG (Sigma) diluted 1 in 1000 with 0.01 M PBS, pH 7.4. Colour was developed by treatment with substrate solution diaminobenzidine hydrochloride (Sigma)

1 mg/ml in 10 mM Tris-HCl, pH 7.5 and containing 200 mM NaCl and H_2O_2 30% at a concentration of 0.3 $\mu\text{l/ml}$.

Inhibition of intestinal adherence in vitro by anti-OMP sera

Adherence activities of different *V. cholerae* strains to isolated rabbit intestinal slices were determined *in vitro* by a radioactive assay system (Dasgupta, 1986) with ^{14}C -labelled vibrios. Bacteria were grown in glucose-free Tryptic Soy Broth (TSB; Difco) containing D- ^{14}C -glucose 1 $\mu\text{Ci/ml}$. The radiolabelled cells were harvested by centrifugation, washed and resuspended in 0.01 M Krebs-Ringer-Tris (KRT) buffer, pH 7.6, to a density of 70–80 Klett units (KU) under a green filter. The bacterial suspension (5 ml) in 10-ml Erlenmeyer flasks was gently shaken with two 1-cm diameter slices of rabbit ileum at 37°C for 10 min. After incubation, each slice of tissue was removed, washed thoroughly in cold KRT, transferred to a scintillation vial and digested with a mixture of perchloric acid and H_2O_2 (Mahin and Lofberg, 1966). After the addition of 5 ml of ethyl cellosolve and 10 ml of toluene-phosphor solution (0.008 g 2,5-diphenyloxazole/ml of toluene) to the vials, radioactivity was counted in a Liquid Scintillation Counter (Model L-50B, Beckman). The radioactivity of 100- μl portions of the suspension of ^{14}C -labelled bacteria treated as above was also measured. Results were expressed as Adhesion Index = $X/Y \times 100$, where X is total counts/adhered slice and Y is counts/ml of interacting bacterial suspension. Adherence inhibition experiments were performed by incubating a mixture of 2.5 ml of ^{14}C -labelled bacteria suspended in KRT buffer and 2.5 ml of antiserum (at a final dilution four times less than the minimum concentration giving cell agglutination) for 30 min at 37°C before addition of tissue slices. Results were expressed as percentage inhibition of intestinal adherence.

Protection experiments

The protective ability of the anti-OMP serum against live vibrio challenge was determined in the rabbit ileal loop assay (De and Chatterje, 1953) by mixing an equal volume of washed *V. cholerae* cells suspended to 200 KU in normal saline with serum, at a dilution two times less than the minimum concentration causing cell agglutination, and injecting 1 ml of the mixture into ligated ileal loops of rabbits. One-ml amounts of *V. cholerae* suspension (100 KU) or normal saline were injected into different loops as positive and negative controls, respectively. Samples of preimmune rabbit serum, at an arbitrarily chosen dilution (1 in 10), and *V. cholerae* suspension were mixed and 1 ml of this mixture was injected into separate loops of rabbit ileum. Animals were killed after 20 h and the accumulated fluid volume (V)/cm of the loop length (L) determined. Antiserum-mediated protection was expressed as the percentage reduction in the V/L ratio in the test loop as compared to that of the positive control.

Results

SDS-PAGE profiles of OM preparations

OM preparations of four strains of *V. cholerae* of serovar O1 of different biotype and serotype and of one strain of *V. cholerae* of non-O1 serovar were subjected to SDS-PAGE. Their OMP profiles are shown in fig. 1. All four strains of serovar O1 showed similar profiles characterised by the presence of a major protein band of 43 Kda. They also shared other proteins of size 47–48, 36, 26–27 (doublet) and 22 Kda. However, the relative amounts of these proteins varied amongst strains. The OMP profile of the non-O1 strain differed somewhat from those of the O1 strains, its major protein being of 38 Kda, whereas other proteins were of size 48, 33, 26–27 and 19–20 (doublet) Kda.

Reactivity of the anti-OMP sera with the OM preparations by ELISA

Antisera raised separately against the OM preparation of strain A17 (serotype Ogawa) of *V. cholerae* of serovar O1 and strain 10259 of a non-O1 serovar were found to contain anti-LPS activity because they formed precipitin bands against their LPS preparations. Both antisera were rendered specific for OMP by absorption with their respective homologous LPS preparations. The absorbed antisera failed to react with the LPS in immunodiffusion or ELISA tests.

The activity of the anti-OMP sera thus prepared

Fig. 1. SDS-PAGE OMP profiles of *V. cholerae*: serovar O1 strains A17 Ogawa, ElTor (lane A), AD29 Inaba, ElTor (B), Vch154 Ogawa, classical (C), 569B Inaba, classical (D), and a non-O1 serovar strain 10259 (E). Protein standards with known mol. wt (Kda) are shown in lane F.

against the OM preparations of different strains of serovars O1 and non-O1 was tested by ELISA. Antiserum to OMP of *V. cholerae* O1 (strain A17 of serotype Ogawa) reacted strongly with the OM preparations of the same strain as well as with preparations from other O1 and non-O1 strains (fig. 2). Similarly, antiserum to OMP of strain 10259 of non-O1 serovar reacted strongly with the OM preparations of that strain as well as with preparations of other O1 strains (data not shown).

Immunoblotting with anti-OMP sera

The reactivity of antiserum to the OMP of *V. cholerae* strain A17 of serotype Ogawa and serovar O1 was tested by immunoblotting against the OM preparations of different O1 and non-O1 strains. The antiserum recognised predominantly two major proteins of 36 and 25–26 Kda present in all four O1 strains, irrespective of biotype and serotype, and also in the non-O1 strain 10259 (fig. 3). Other proteins of > 36 Kda and < 25 Kda reacted weakly with the antiserum.

Antiserum to the OMP of strain 10259 of non-O1 serovar recognised predominantly proteins of 36 and 25–26 Kda in the OM preparations of different O1 strains and proteins of 36–38 and 25–

Fig. 2. ELISA titres of the antiserum to *V. cholerae* O1 (strain A17, serotype Ogawa) OMP determined against the OM preparations of *V. cholerae* strains of serovars O1 (curves 1–4) and non-O1 (curve 5). Curve 6 shows the reactivity of the pre-immune rabbit serum to the OM of strain A17 Ogawa. ●—● Strain A17 Ogawa; ○—○ strain Vch154 Ogawa; □—□ strain AD29 Inaba; △—△ strain 569B Inaba; ×—× strain 10259 (non-O1).

Fig. 3. Immunoblot analysis of anti-OMP serum (raised against strain A17, Ogawa) against OM preparations of *V. cholerae* serovar O1 strains A17 Ogawa (lane A), AD29 Inaba (B), Vch154 Ogawa (C), 569B Inaba (D) and a non-O1 strain 10259 (E). Lane F shows the reactivity of pre-immune serum to the OM of strain A17 Ogawa.

26 Kda in the OM preparation of the homologous strain 10259 (fig. 4).

Agglutinability of anti-OMP sera for *V. cholerae* O1 and non-O1 whole cells

Antiserum to the OMP of strains of *V. cholerae* of serovar O1 agglutinated all 14 strains of *V. cholerae* of serovar O1 and 14 (84%) of 17 strains of non-O1 serovars tested (table I). Similarly, antiserum to the OMP of the non-O1 strain 10259 agglutinated 10 (91%) of 11 strains of O1 serovar and 20 (87%) of 23 non-O1 strains tested (table I).

Fig. 4. Immunoblot analysis of anti-OMP serum (10259) against OM preparations of *V. cholerae* serovar O1 strains A17 Ogawa (lane A), AD29 Inaba (B), Vch154 Ogawa (C), 569B Inaba (D) and a non-O1 strain 10259 (E). Lane F shows the reactivity of pre-immune serum to the OM of the non-O1 strain 10259.

Table I. Agglutinability of strains of *V. cholerae* of O1 and non-O1 serovars by antiserum to OMP

Antiserum raised against OM preparation of <i>V. cholerae</i> strain*	Number of strains of <i>V. cholerae</i> of					
	serovar O1			non-O1 serovar		
	tested	agglutinated	not agglutinated	tested	agglutinated	not agglutinated
A17 Ogawa (O1)	14	14	0	17	14	3
10259 (non-O1)	11	10	1	23	20	3

* Antiserum to OM was absorbed with homologous LPS preparation to render it specific for OMP.

Anti-adherence activities of the anti-OMP sera

When antiserum to OMP of *V. cholerae* strain A17 (serotype Ogawa, serovar O1) was tested at a concentration four times more dilute than the minimum concentration causing cell agglutination, it reduced the adhesion index of the parent organism by 48% (table II). This antiserum also inhibited to a moderate (32–40%) degree the adhesion of other O1 and non-O1 strains to rabbit ileum under comparable conditions. Similarly, antiserum to the OMP of the non-O1 strain 10259 reduced the adhesion index of the homologous strain by 49% and that of strain A17 (serotype Ogawa, serovar O1) by 38% (data not shown).

Protective activity of the anti-OMP serum

When antiserum to the OMP of *V. cholerae* strain

Table II. Anti-adhering activities of antiserum* to outer membrane proteins of *V. cholerae* strain A17 of serotype Ogawa and serovar O1

Adhering <i>V. cholerae</i> strain	Adhesion index† in test made in presence of		Inhibition (%)
	KRT buffer (control)	OMP antiserum‡	
A17 Ogawa (O1)	9.0	4.7	48
AD29 Inaba (O1)	12.0	7.8	35
569 Inaba (O1)	2.5	1.7	32
Vch154 Ogawa (O1)	2.0	1.2	40
10259 (non-O1)	14.4	9.5	34

* Antiserum was raised against the OM preparation of *V. cholerae* O1 (strain A17, serotype Ogawa, serovar O1) and absorbed with homologous LPS preparation to render it specific for OMP.

† Mean values of duplicate sets are shown.

‡ Antiserum was used at a concentration four times more dilute than the minimum concentration causing cell agglutination.

A17 (serotype Ogawa, serovar O1) was tested at a concentration four times more dilute than the minimum concentration causing cell agglutination, it gave some protection (55%) against challenge with the homologous strain in the ligated ileal loops of rabbits (table III). The antiserum also exhibited a moderate degree of protection (38–45%) against challenge with other O1 and non-O1 strains. Such protection was not found in tests with pre-immune rabbit serum.

Discussion

In this study, remarkable similarities were noted between the OMP profiles of different strains of *V. cholerae* of serovar O1. However, the OMP profile of a non-O1 strain showed some differences; the major OMP of the non-O1 strain was about 38 Kda whereas that of the O1 strains were all of identical size, 43 Kda. When less sample was loaded on to gels, the major OMP was found to consist of more than one protein of closely similar mol. wt (data not shown). Similar results were obtained previously by other workers (Manning *et al.*, 1982; Sciortino and Finkelstein, 1983) who showed that the major protein complex possessed porin-like properties in that it was trypsin resistant and peptidoglycan associated. Kabir (1980, 1983) reported that the major cell-surface protein of *V. cholerae* O1 had a subunit mol. wt of 48 Kda and was immunogenic. The protein could be detected in strains of all biotypes and serotypes of *V. cholerae*. On the other hand, Manning and Haynes (1984) reported that a 25-Kda OMP was markedly immunogenic and common to all *Vibrio* strains tested by them.

Here, the antigenic relatedness between some OMPs of *V. cholerae* O1 and non-O1 strains was clearly established. Immunoblotting experiments revealed that at least two major immunogenic

Table III. Protective activity of antiserum to outer membrane proteins of *V. cholerae* strain A17 of serotype Ogawa and serovar O1

Challenge strain of <i>V. cholerae</i>	Pre-incubated with	Fluid accumulation ratio (V/L)	Protection (%)
A17 Ogawa (O1)	Normal saline	0.88	55
	Pre-immune serum*	0.8	
	Anti-OMP serum†	0.4	
569B Inaba (O1)	Normal saline	1.0	45
	Pre-immune serum*	1.1	
	Anti-OMP serum†	0.55	
10259 (non-O1)	Normal saline	0.8	38
	Pre-immune serum*	0.8	
	Anti-OMP serum†	0.5	

* Pre-immune serum was used at an arbitrarily chosen dilution (1 in 20).

† Antiserum to the OM preparation of strain A17, serotype Ogawa was absorbed with the homologous LPS to render it specific for OMP. Antiserum was used at a concentration four times more dilute than the minimum concentration causing cell agglutination.

OMPs of 36 and 25–26 Kda were common to all four strains of *V. cholerae* of serovar O1 examined, irrespective of their biotype and serotype. Antigenically-related bands of similar mol. wt were also demonstrable in the non-O1 strain 10259. In addition to these two proteins, there might be other antigenically common OMPs in *V. cholerae* O1 and non-O1 strains although these were probably less immunogenic, at least in rabbits. In blotting experiments the major OMP complex of 43 Kda showed very weak reactivity, even with the homologous OM preparation. Recently, we purified this major OMP from SDS-polyacrylamide gels. Antiserum to it raised in rabbits recognised only the 43-Kda protein(s) in immunoblotting experiments (D. Sengupta and A. C. Ghose, unpublished observation) and agglutinated all *V. cholerae* O1 strains but only 25% of non-O1 strains tested.

Cell-surface proteins are believed to play an important role in the intestinal adhesion and subsequent colonisation process of *V. cholerae*. The finding that antibodies to OMPs could inhibit, albeit partially, the intestinal adhesion of *V. cholerae* supports this view. Furthermore, our results also

suggest that some of the OMPs critical for adhesion are immunogenic and probably shared by *V. cholerae* strains irrespective of biotype, serotype and serovar. However, inability of anti-OMP serum to inhibit completely the intestinal attachment of *V. cholerae* is indicative of the possible involvement of non-OMP components also in the adhesion process.

Sears *et al.* (1984) demonstrated antibodies to OMPs in human sera after ingestion of *V. cholerae* by volunteers. Stimulation of antibody response to OMPs was also noted by us in sera from cholera patients (D. Sengupta and A. C. Ghose, unpublished data). That such antibodies may have some protective significance is supported by the data presented here (table III). Similar results were also obtained by Sharma *et al.* (1987) who showed the prophylactic significance of non-LPS components of *V. cholerae* of serovar O1 in an infant mouse model. Finally, the antiserum-mediated cross-protection observed in this study between *V. cholerae* O1 and non-O1 serovars strengthens the argument that OMPs be included in the design and development of a vaccine against cholera.

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