INFECTION AND IMMUNITY, Nov. 1992, p. 4848–4855
0019-9567/92/114848-08$02.00/0
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Vol. 60, No. 11

Major Outer Membrane Proteins of *Vibrio cholerae* and Their Role in Induction of Protective Immunity through Inhibition of Intestinal Colonization

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Vibrio cholerae **O1 organisms belonging to different biotypes and serotypes were shown to express major outer membrane proteins (MOMPs) with subunit molecular masses of 48 to 50, 40 to 43, 35 to 36, 27 to 28, and 20 kDa. Antisera raised against individual MOMPs of a** ***V. cholerae*** **O1 strain recognized MOMPs of corresponding molecular masses in other O1 and non-O1 strains. Serological data also suggested possible differences in the cell surface exposition of these MOMPs. However, no marked differences between** ***V. cholerae*** **cells grown in vitro and in vivo could be noted in respect to the expression or surface exposition of these MOMPs. Of five MOMPs studied in this work, 40- to 43- and 20-kDa cell surface proteins were shown to be of considerable importance, as antisera to these proteins induced significant protection against** ***V. cholerae*** **challenge in the suckling mouse model. Similar protection, although to a lesser extent, was demonstrable with the antiserum to the 27- to 28-kDa protein. These results were corroborated with the Fab (immunoglobulin G) [Fab(IgG)] fragments of the antisera, thereby suggesting that the observed protection induced by anti-MOMP antibodies did not arise as a result of bacterial clumping. Subsequent studies demonstrated that these antisera as well as their Fab(IgG) fragments induced significant inhibition of intestinal colonization of** ***V. cholerae*****. The 40- to 43- and 27- to 28-kDa proteins appeared to be porinlike, while the 20-kDa protein was found to be antigenically related to TcpA (subunit A of toxin-coregulated pilus). All these results demonstrate the involvement of more than one cell surface antigen of** ***V. cholerae*** **in the induction of protective immunity through inhibition of intestinal colonization of vibrios.**

The enteropathogenic organism *Vibrio cholerae* causes cholera in humans through intestinal colonization and elaboration of a potent enterotoxin, also known as cholera toxin (15, 23). Although antitoxic immunity plays an important role in protection against cholera, antibacterial immunity appears to be of even greater importance (24). Serum antibodies directed against *V. cholerae* somatic antigens are usually vibriocidal in nature (13, 25). However, the same may not be true for the secretory immunoglobulin A (IgA) class of antibodies present in intestinal and other body secretions (26). The secretory IgA class of antibodies is believed to act through inhibition of intestinal attachment and subsequent colonization of vibrios (27). However, identification of cell surface moieties of *V. cholerae* responsible for attachment of the organism to intestinal epithelium has remained elusive so far.

Antibacterial response in cholera has been shown to be directed against both lipopolysaccharide (LPS) (24, 25) and outer membrane (OM) proteins (OMPs) of *V. cholerae* (35, 37). Despite the fact that antibodies to *V. cholerae* LPS are known to mediate protection against cholera (4, 10, 16), the existence of non-LPS protective antigens has also been documented (1, 42). In fact, in recent years, there has been considerable interest in studying the cell surface proteins of *V. cholerae* and evaluating their role as protective antigens (29, 38, 42). We have earlier demonstrated that the antibodies to *V. cholerae* OMPs play an important role in protection which is likely to be mediated through inhibition of intestinal colonization of vibrios (39). In the present work, we have further extended this observation by studying the major OMPs (MOMPs) of *V. cholerae* on an individual basis and attempted to identify the OMPs responsible for the induction of protective immunity in the host through inhibition of intestinal colonization of vibrios.

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MATERIALS AND METHODS

Bacterial strains. *V. cholerae* strains of both O1 and non-O1 serovars were used in this study. These strains were isolated from diarrheal stool samples as described earlier (7, 38) and maintained in nutrient agar stabs or slants by serial passages.

In vitro and in vivo growth conditions of *V. cholerae*. Different *V. cholerae* strains were grown in vitro in Erlenmeyer flasks containing Trypticase soy broth (TSB) (HiMedia, Bombay, India). An early-logarithmic-phase seed culture was used as inoculum, and bacteria were grown at 37°C for 18 h with shaking.

Bacterial cells grown in vitro were washed and suspended in normal saline to give a suspension containing 10^6 organisms per ml. One milliliter of this suspension was injected into washed and ligated intestinal segments (5 to 6 cm in length each) of adult rabbits which were kept alive for 16 to 18 h. Next, rabbits were sacrificed, and fluid accumulated in the intestinal segments was collected by a syringe. Materials were centrifuged at 3,000 × *g* for 5 min to remove debris. Finally, *V. cholerae* cells were pelleted from the supernatant by centrifugation at 8,000 × *g* for 10 min. The pellet was washed with normal saline and stored at 4°C for 24 to 48 h before use. All procedures were performed at 4°C.

Preparation of OM. OMs were prepared from different

V. cholerae strains grown in vitro under the conditions described above. The method of Filip et al. (9) was followed with minor modifications (38). Briefly, harvested bacteria were washed and suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Sigma Chemical Co., St. Louis, Mo.), pH 7.0. Next, cells were disrupted in an ultrasonic disintegrator (B. Braun, Melsungen AG) at 4°C, and intact cells remaining were removed by low-speed centrifugation (6,000 × *g* for 10 min). The crude envelope fraction was collected from the supernatant by centrifugation at 105,000 × *g* for 1 h at 4°C. The pellet containing the crude envelope fraction was treated with 0.5% (wt/vol) Sarkosyl (Sigma) solution to selectively solubilize the inner membrane part. The insoluble OM fraction was recovered as pellet by centrifugation at 105,000 × *g* for 1 h at 4°C. The pellet was washed and stored at −20°C until used. Protein contents of OM preparations were determined by the method of Markwell et al. (32) with bovine serum albumin (BSA) (Sigma) as standard.

Preparation of LPS. LPS were extracted from acetone-dried cells of *V. cholerae* O1 strain 154 (classical, Ogawa) by the phenol-water extraction method (50). Residual protein in the LPS preparation was removed by proteolytic digestion (38). Digested material was extensively dialyzed against distilled water and lyophilized.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were analyzed on 12.5% (wt/vol) polyacrylamide gels with 4.5% (wt/vol) stacking gels on top (22). Membrane preparations containing about 80 to 100 μg of protein were solubilized in sample buffer containing 2% (wt/vol) SDS and 5% (vol/vol) β-mercaptoethanol, heated at 100°C for 10 min, and electrophoresed at room temperature at constant voltage (120 V) for 4 h. Following electrophoresis, gels were fixed and stained with Coomassie blue R250 (Sigma). Low-molecular-weight protein standards (Sigma) were used for molecular weight determination.

Raising of antisera to individual MOMPs. Antisera to individual MOMPs (48 to 50, 40 to 43, 35 to 36, 27 to 28, and 20 kDa) of *V. cholerae* O1 strain 154 were raised in rabbits. For this, OM samples were first electrophoresed in an SDS-polyacrylamide gel, and following electrophoresis, the gel was immediately placed in a chilled (4°C) potassium chloride solution (1 M). Protein bands became visible against an opaque background within 20 to 30 min (34). Gels thus developed were appropriately cut to obtain fragments containing the desired MOMP only. Next, the gel fragments were suspended in 1 ml of saline, homogenized, and injected intramuscularly into different rabbits. Five such injections were administered at fortnight intervals, and sera were collected. Sera were decomplemented at 56°C for 30 min. Any anti-LPS activity present in the anti-MOMP serum was removed by absorption with purified LPS (38) prepared from O1 strain 154. Efficacy of the anti-LPS absorption procedure was determined by enzyme-linked immunosorbent assay (ELISA) of the absorbed antiserum against LPS (25).

Preparation of Fab(IgG) fragment. IgG fraction of rabbit serum was prepared by the salt fractionation procedure (17). The fraction was digested with papain (Sigma), and Fab fragments were isolated from the digested material by carboxymethyl cellulose column chromatography (44).

ELISA. The antibody titer of anti-MOMP serum was determined by ELISA in microtiter plates with *V. cholerae* whole cells (6) or their OM preparations (38) as antigen. Titer was expressed as the highest dilution of the antiserum that showed definite color development (an optical density of ≥0.2 at 490 nm) in the assay.

Immunoblot analysis. Immunochemical detection of antigen on nitrocellulose paper was carried out by the method of Towbin et al. (47) with minor modifications (38). For this, proteins were subjected to SDS-PAGE and then electrophoretically transferred to nitrocellulose strips. Next, blotted strips were treated with 3% (wt/vol) BSA and then incubated with appropriate dilutions of absorbed antisera. The strips were then washed, treated with peroxidase-labeled goat anti-rabbit IgG (Sigma), and developed with the substrate solution containing diaminobenzidine hydrochloride (Sigma) and H_2O_2.

Bacterial agglutination. *V. cholerae* organisms were grown in TSB at 37°C, harvested, washed, and finally suspended in Krebs-Ringer-Tris (KRT) buffer (0.01 M Tris base, 0.13 M NaCl, 5.14 mM KCl, 1.29 mM $MgSO_4$, 2.74 mM $CaCl_2$, pH 7.4) to a density of 10^{10} cells per ml. One hundred microliters of twofold serially diluted antiserum was added to an equal volume of the bacterial suspension in glass tubes, and the mixtures were incubated for 2 h at 37°C and then overnight at 4°C. The agglutination pattern was recorded, and the titer was expressed as the highest dilution of the antiserum showing a positive agglutination reaction.

Protection experiments in suckling mouse model. The protective activity of anti-MOMP serum or its Fab(IgG) preparation was determined by passive protection experiments in a suckling mouse model (48). For this, each group of animals was inoculated orally with 0.1 ml of vibrio suspension (10 times the 50% lethal dose [LD_{50}] as determined by earlier experiments) preincubated with appropriate dilutions of anti-MOMP serum or its Fab(IgG) preparation. *V. cholerae* organisms grown in TSB at 37°C for 18 h with shaking were used for all these experiments. A group of mice which received vibrio suspension alone (without any antiserum) served as the negative control. Results were expressed as percentage of protection by comparing the number of survivors after 24 h with the total number of mice used in each experimental group.

Inhibition of intestinal colonization of *V. cholerae*. Inhibition of intestinal colonization of *V. cholerae* in the suckling mouse was studied (39). For this, each group of animals (three or four mice per group) was challenged orally with 0.1 ml of vibrio suspension (containing approximately 2.5×10^5 CFU of highly colonizing *V. cholerae* El Tor, Ogawa strain A17) which was preincubated at 37°C for 30 min with appropriate dilutions of anti-MOMP serum or its Fab(IgG) preparation. After 14 to 16 h of challenge, animals were sacrificed, and their intestines were removed and washed in normal saline to remove nonadherent bacteria. Next, the intestines were homogenized separately by using a homogenizer (REMI, Bombay, India). Viable counts in each homogenate were determined by the plate count method. A group of mice which received vibrio suspension alone (without any antiserum) served as the negative control.

RESULTS

SDS-PAGE profiles of OM preparations. The protein profiles of OM preparations of *V. cholerae* strains were analyzed by SDS-PAGE (Fig. 1). All the strains belonging to serovar O1 showed similar profiles characterized by the presence of intensely stained protein band(s) in the molecular mass range of 40 to 43 kDa. The band appeared to be composed of more than one subunit, all with very similar molecular masses. The O1 strains also shared other MOMPs of subunit molecular masses 48 to 50, 35 to 36, 27 to 28 (doublet), and 20 kDa. However, the relative amounts of

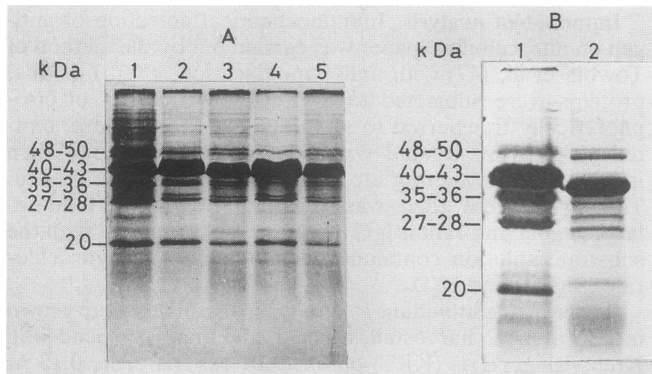

FIG. 1. SDS-PAGE profiles of OMPs of *V. cholerae* O1 and non-O1 strains grown in TSB at 37°C. (A) Serovar O1 strains. Lane 1, 154; lane 2, 569B; lane 3, A17; lane 4, AD20; lane 5, AD29. (B) Serovar non-O1 strains. Lane 1, V_2; lane 2, 10259.

these proteins varied among different strains. The MOMP profiles of non-O1 strains were very similar to those of O1 strains except for non-O1 strain 10259, which had the MOMP band at around 38 kDa instead of 40 to 43 kDa (Fig. 1).

Reactivities of anti-MOMP sera to OM preparations. Antisera raised against the individual MOMPs (48 to 50, 40 to 43, 35 to 36, 27 to 28, and 20 kDa) of *V. cholerae* O1 strain 154 were tested to determine their reactivities against OM preparations from the parent strain as well as from other *V. cholerae* O1 and non-O1 strains. Results (Fig. 2) showed that anti-MOMP sera recognized protein bands of identical subunit molecular masses in the OM preparations of *V. cholerae* strains belonging to different biotypes, serotypes, and even serovars. One exception was the non-O1 strain 10259, the OM preparation of which failed to react with anti-20-kDa-protein serum, although it reacted with the other four

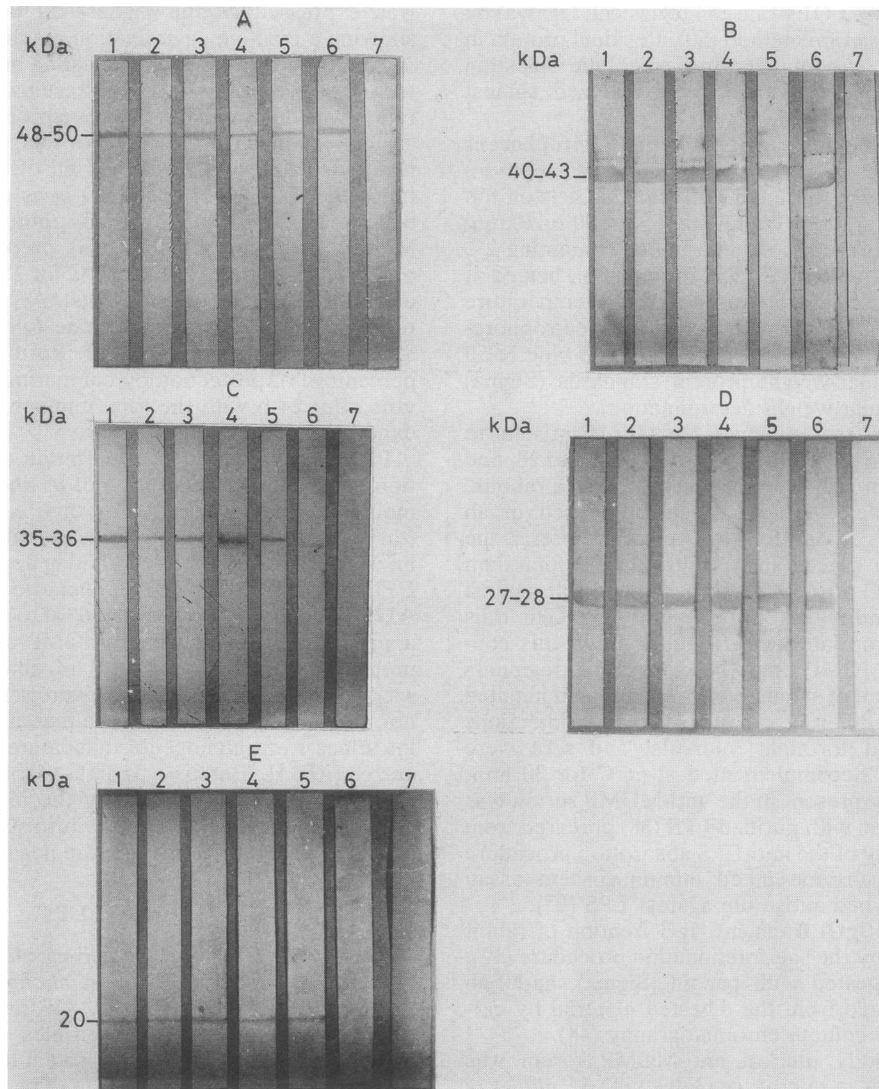

FIG. 2. Immunoblot analysis of OM preparations of *V. cholerae* O1 (lanes 1 to 5) and non-O1 (lane 6) strains. Lanes 1, A17; lanes 2, AD20; lanes 3, AD29; lanes 4, 154; lanes 5, 569B; lanes 6, 10259. Rabbit antisera to MOMPs of molecular masses 48 to 50 kDa (A), 40 to 43 kDa (B), 35 to 36 kDa (C), 27 to 28 kDa (D), and 20 kDa (E) were used. Lanes 7 show the reactivity of preimmune rabbit sera against OM preparation of *V. cholerae* 154.

TABLE 1. Reactivities of anti-MOMP sera[a] against OM preparations of O1 and non-O1 *V. cholerae* strains

V. cholerae strain	Serovar	Biotype and serotype	ELISA titer[b] (reciprocal) of antiserum to MOMP				
			48–50 kDa	40–43 kDa	35–36 kDa	27–28 kDa	20 kDa
154	O1	Classical, Ogawa	5,120	20,480	20,480	10,240	10,240
569B	O1	Classical, Inaba	2,560	10,240	10,240	5,120	5,120
A17	O1	El Tor, Ogawa	5,120	20,480	20,480	10,240	5,120
AD29	O1	El Tor, Inaba	2,560	10,240	10,240	5,120	5,120
AD20	O1	El Tor, Ogawa	2,560	10,240	5,120	10,240	10,240
10259	Non-O1	59 (Smith)	2,560	10,240	10,240	5,120	≤80
V_2	Non-O1	37 (Sakazaki)	2,560	5,120	2,560	2,560	5,120

[a] Antisera to individual MOMPs of *V. cholerae* 154 were raised separately in rabbits. These were subsequently absorbed with the LPS preparation of the same strain.
[b] ELISAs were carried out by using goat anti-rabbit IgG-peroxidase conjugate. A control optical density of 0.2 at 490 nm was used as the cutoff value for determination of ELISA titers.

anti-MOMP sera. These results were also corroborated by ELISA, which demonstrated the presence of antigenically related MOMPs in different *V. cholerae* O1 and non-O1 strains (Table 1).

Reactivities of anti-MOMP sera to whole cells of *V. cholerae*. Antisera to individual MOMPs were tested to check their agglutinating activities toward whole cells of *V. cholerae*. Results (Table 2) show that antiserum to 40- to 43-kDa proteins agglutinated 9 of 10 O1 and 5 of 8 non-O1 *V. cholerae* strains. On the other hand, antiserum to 20-kDa protein agglutinated 8 of 10 O1 and 2 of 8 non-O1 strains tested. However, antisera to other MOMPs (48 to 50, 35 to 36, and 27 to 28 kDa) failed to show any detectable agglutinating activity towards the different *V. cholerae* strains, including parent strain 154 (Table 2). Next, the reactivities of these anti-MOMP sera were tested in ELISAs against whole cells of *V. cholerae* O1 strains 154 (classical, Ogawa) and A17 (El Tor, Ogawa) grown in vitro and in vivo (obtained by differential centrifugation of the fluid accumulated in rabbit ileal loops). Only weak reactivity was observed with the three anti-MOMP (48 to 50, 35 to 36, and 27 to 28 kDa) sera compared with that of anti-40-to-43- and anti-20-kDa-MOMP sera (Table 3).

Protective activities of anti-MOMP sera and their Fab(IgG) preparations. The protective activities of anti-MOMP sera against live vibrio challenge were tested by passive protection experiments in the suckling mouse model (Tables 4 and 5). It is evident that antisera to the 40- to 43- and 20-kDa cell surface proteins induced marked protection not only against challenge with immunizing strain 154 (Table 4) but also against *V. cholerae* A17 of a heterologous biotype (Table 5). Such protection was also demonstrable even at subagglutinating dilutions of the two antisera. A moderate degree of protection against vibrio challenge could also be induced by the antiserum corresponding to the 27-to-28-kDa protein. However, only marginal protection if any could be observed with antisera to other MOMPs (48 to 50 and 35 to 36 kDa).

Results (Tables 4 and 5) obtained with three of the anti-MOMP sera, which showed significant protection against vibrio challenge, were further extended by using their Fab(IgG) fragments. It is apparent (Table 6) that Fab(IgG) fragments of antisera to the 40- to 43- and 20-kDa MOMPs induced a strong or moderate degree of protection, respectively, against challenge with parent strain 154, while the Fab(IgG) of anti-27-to-28-kDa-MOMP serum induced only weak protection under similar conditions. No such protection could be demonstrated with the Fab(IgG) fragment of normal rabbit serum (Table 6). The concentrations of Fab(IgG) fragments used in protection experiments (300 and 100 μg/ml) were arbitrary.

Inhibition of intestinal colonization of *V. cholerae* by anti-MOMP sera and their Fab(IgG) preparations. The three anti-MOMP (40 to 43, 27 to 28, and 20 kDa) sera which induced significant protection against vibrio challenge in earlier experiments were further tested to evaluate their capacities to inhibit intestinal colonization of highly colonizing *V. cholerae* A17. It is evident (Table 7) that all three anti-MOMP sera significantly inhibited intestinal coloniza-

TABLE 2. Agglutinabilities of different strains of *V. cholerae* by anti-MOMP sera[a]

MOMP (kDa)	Agglutination of *V. cholerae*			
	No. of O1 strains (n = 10)		No. of non-O1 strains (n = 8)	
	Agglutinated	Not agglutinated	Agglutinated	Not agglutinated
48–50	0	10	0	8
40–43	9	1	5	3
35–36	0	10	0	8
27–28	0	10	0	8
20	8	2	2	6

[a] Antisera to individual MOMPs of *V. cholerae* 154 were raised separately in rabbits. These were subsequently absorbed with the LPS preparation of the same strain.

TABLE 3. Reactivities of anti-MOMP sera[a] against whole cells of *V. cholerae* grown in vivo and in vitro

MOMP (kDa)	ELISA titer[b] (reciprocal) of antiserum against:			
	A17 (Ogawa, El Tor)		154 (Ogawa, classical)	
	In vivo[c]	In vitro[d]	In vivo	In vitro
48–50	320	640	640	640
40–43	2,560	10,240	5,120	10,240
35–36	160	320	320	640
27–28	320	1,280	640	640
20	1,280	2,560	1,280	5,120

[a] Antisera to individual MOMPs of *V. cholerae* 154 were raised separately in rabbits. These were subsequently absorbed with the LPS preparation of the same strain.
[b] ELISAs were carried out by using goat anti-rabbit IgG-peroxidase conjugate. A control optical density of 0.2 at 490 nm was used as the cutoff value for the determination of ELISA titers.
[c] Bacteria were harvested by differential centrifugation from fluids accumulated in rabbit ileal loops by 18 h after inoculation with *V. cholerae* strains.
[d] Bacteria were grown in TSB at 37°C for 18 h with shaking.

TABLE 4. Protective activities of anti-MOMP sera[a] against challenge with *V. cholerae* O1 strain 154 (Ogawa, classical) in suckling mouse model[b]

Preincubation serum[c]	Antiserum dilution	% Protection[d]
Preimmune	1:5	0
Anti-MOMP (48–50 kDa)	1:10	33.3
	1:20	14.3
Preimmune	1:5	0
Anti-MOMP (40–43 kDa)	1:80	87.5
	1:160	83.3
Preimmune	1:5	0
Anti-MOMP (35–36 kDa)	1:20	14.3
	1:40	0
Preimmune	1:5	0
Anti-MOMP (27–28 kDa)	1:20	57.1
	1:40	33.3
Preimmune	1:5	0
Anti-MOMP (20 kDa)	1:40	66.6
	1:80	42.9

[a] Rabbit antisera to individual MOMPs of *V. cholerae* O1 strain 154 were raised separately in rabbits. These antisera were absorbed by the LPS preparation of the same strain.
[b] Each mouse was challenged with 10 LD_{50}s (approximately 8×10^8 CFU) of bacteria suspended in 0.1 ml of normal saline containing appropriate dilutions of antisera.
[c] Preimmune serum did not show any agglutination of *V. cholerae*. Two- and fourfold subagglutinating dilutions of anti-MOMP sera were used.
[d] Six to eight mice were included in each group of animals, and percent protection was expressed as the percentage of survivors in each group.

tion by A17 even at subagglutinating dilutions. However, considerable variations in their inhibitory capacities were apparent. Thus, anti-40-to-43-kDa-MOMP serum exhibited more than 1,000-fold inhibition of vibrio colonization at 4-fold subagglutinating dilutions, while anti-27-to-28-kDa-MOMP serum showed only about 20-fold inhibition under similar conditions. The preimmune sera, however, failed to show any such inhibitory activity. These data were further extended by using Fab(IgG) fragments of the three anti-MOMP sera (Table 8). The Fab(IgG) fragment of anti-40-to-43-kDa-MOMP serum showed the highest inhibitory capacity; the anti-20-kDa-MOMP serum showed the second highest inhibitory capacity. The Fab(IgG) of anti-27-to-28-kDa-MOMP serum, however, showed only marginal inhibitory activity under similar experimental conditions.

TABLE 5. Protective activities of anti-MOMP sera[a] against challenge with *V. cholerae* O1 strain A17 (Ogawa, El Tor) in suckling mouse model[b]

Preincubation serum[c]	Antiserum dilution	% Protection[d]
Preimmune	1:5	0
Anti-MOMP (48–50 kDa)	1:10	33.3
	1:20	16.6
Preimmune	1:5	0
Anti-MOMP (40–43 kDa)	1:80	87.5
	1:160	75
Preimmune	1:5	0
Anti-MOMP (35–36 kDa)	1:20	12.5
	1:40	0
Preimmune	1:5	0
Anti-MOMP (27–28 kDa)	1:20	50
	1:40	25
Preimmune	1:5	0
Anti-MOMP (20 kDa)	1:80	62.5
	1:160	37.5

[a] Rabbit antisera to MOMPs of *V. cholerae* O1 strain 154 were absorbed by the LPS preparation of the same strain.
[b] Each mouse was challenged with 10 LD_{50}s (approximately 2×10^8 CFU) of bacteria suspended in 0.1 ml of normal saline containing appropriate dilutions of antisera.
[c] Preimmune serum did not show any agglutination of *V. cholerae*. Two- and fourfold subagglutinating dilutions of anti-MOMP sera were used.
[d] Six to eight mice were included in each group of animals, and percent protection was expressed as the percentage of survivors in each group.

TABLE 6. Protective activities of Fab(IgG) fragments of anti-MOMP sera[a] against *V. cholerae* 154 (Ogawa, classical) challenge in suckling mouse model[b]

Preincubation fluid	Concn of Fab (μg/ml)	% Protection[c]
Normal saline		0
Fab fragments		
Normal rabbit serum	300	0
Anti-MOMP (40–43 kDa) serum	300	75
	100	62.5
Anti-MOMP (27–28 kDa) serum	300	33.3
	100	16.6
Anti-MOMP (20 kDa) serum	300	57
	100	42.8

[a] Rabbit antisera to MOMPs of *V. cholerae* O1 strain 154 were absorbed by the LPS preparation of the same strain.
[b] Each mouse was challenged with 10 LD_{50}s (approximately 8×10^8 CFU) of bacteria suspended in 0.1 ml of normal saline containing Fab(IgG) preparation.
[c] Six to eight mice were included in each group of animals, and the percent protection was expressed as the percentage of survivors in each group.

DISCUSSION

Sharing of antigenically related OMPs by different *V. cholerae* O1 strains was documented by earlier workers (21, 30, 38, 42). In this study, we were able to demonstrate that the five MOMPs of subunit molecular masses 48 to 50, 40 to 43, 35 to 36, 27 to 28, and 20 kDa were shared by different *V. cholerae* strains belonging to different biotypes, serotypes, and even serovars. One exception was that the non-O1 strain 10259 had a 22-kDa protein which was antigenically unrelated to the 20-kDa protein present in other O1 and non-O1

TABLE 7. Inhibition of intestinal colonization of *V. cholerae* A17 (Ogawa, El Tor) by anti-MOMP sera[a] in suckling mouse model[b]

Preincubation fluid[c]	Dilution of antiserum	CFU/intestine[d]
Normal saline		2.9×10^7
Preimmune serum	1:5	1.4×10^7
Anti-MOMP serum (40–43 kDa)	1:80	3.8×10^3
	1:160	1.2×10^4
Preimmune serum	1:5	2.2×10^7
Anti-MOMP serum (27–28 kDa)	1:20	9.6×10^4
	1:40	1.4×10^6
Preimmune serum	1:5	1.6×10^7
Anti-MOMP serum (20 kDa)	1:80	3.1×10^4
	1:160	7.2×10^5

[a] Rabbit antisera to MOMP of *V. cholerae* O1 strain 154 were absorbed by the LPS preparation of the same strain.
[b] Each mouse was challenged with 2.2×10^5 CFU of bacteria in 0.1 ml of normal saline with or without anti-MOMP sera.
[c] Preimmune serum did not show any agglutination of *V. cholerae*. Two- and fourfold subagglutination doses of anti-MOMP sera were used.
[d] Determined after 14 h of vibrio challenge. Results are means per group of three or four mice.

TABLE 8. Inhibition of intestinal colonization of *V. cholerae* A17 (Ogawa, El Tor) by Fab(IgG) fragments of anti-MOMP sera[a] in suckling mouse model[b]

Preincubation fluid	Concn of Fab (μg/ml)	CFU/intestine[c]
Normal saline		3.1×10^7
Fab(IgG) fragments[d]		
Normal rabbit serum	300	2.6×10^7
Anti-MOMP (40–43 kDa) serum	300	9.2×10^3
	100	8×10^4
Anti-MOMP (27–28 kDa) serum	300	9.1×10^5
	100	2.1×10^7
Anti-MOMP (20 kDa) serum	300	8.9×10^4
	100	4.3×10^5

[a] Rabbit antisera to MOMPs of *V. cholerae* O1 strain 154 were absorbed by the LPS preparation of the same strain.
[b] Each mouse was challenged with 2.8×10^5 CFU of bacteria in 0.1 ml of normal saline with or without Fab(IgG) fragments of anti-MOMP sera.
[c] Determined after 14 h of vibrio challenge. Results are means per group of three or four mice.
[d] Concentrations used were arbitrary.

strains. Our results also show that antibody responses against all the MOMPs can be induced by separate immunization of the proteins, although antiserum raised against the whole cells of *V. cholerae* (41) or their OM preparations (38) or sera obtained from cholera patients (35, 37) may not contain antibodies against all these MOMPs. Admittedly, the MOMPs used for immunization in this study were less likely to retain their native structures under the conditions of their isolation. However, antisera thus raised showed adequate reactivities to the MOMPs in their native form, as is evident from the ELISA results. Serological data (Tables 1 and 3) also provide some information regarding the relative exposition of MOMPs on the *V. cholerae* surface. Thus, it is tempting to speculate that the 40- to 43- and 20-kDa proteins are relatively more accessible to antibodies than the other three MOMPs (48 to 50, 35 to 36, and 27 to 28 kDa). This is also consistent with the idea that gram-negative bacteria, including *V. cholerae*, have fewer surface-exposed proteins (5, 36, 49), probably as a result of a shielding effect of the long LPS O side chain. Interestingly, all the anti-MOMP sera showed comparable ELISA titers against in vivo- and in vitro-grown cells of *V. cholerae*, thereby suggesting no marked differences in the expression or surface exposition of MOMPs in *V. cholerae* cells grown in vitro and in vivo.

Of the five MOMPs studied here, antisera raised against 40- to 43- and 20-kDa proteins and, to a lesser extent, antiserum to 27- to 28-kDa protein protected mice against *V. cholerae* challenge. That the protection was demonstrable even at subagglutinating dilutions of the antisera or their Fab(IgG) fragments clearly suggests that the observed protection induced by antibodies against these MOMPs did not arise as a result of bacterial clumping. These results were also corroborated by colonization inhibition data, which demonstrated that subagglutinating dilutions of antisera as well as their Fab(IgG) fragments induced significant inhibition of intestinal colonization of *V. cholerae*.

The 40- to 43-kDa MOMP is the most predominant OMP of *V. cholerae* and is probably composed of more than one subunit, all with very similar molecular masses (31). In agreement with the earlier report (31), this protein complex and then 27- to 28-kDa protein were shown to be trypsin resistant and peptidoglycan associated and therefore porinlike (31, 40). Thus, the porin proteins, particularly the 40- to 43-kDa ones, may be considered major protective antigens of *V. cholerae*. Porins of other gram-negative bacteria were also shown to act as protective antigens (11, 28, 33), but no such information is yet available for *V. cholerae*.

Our results show that apart from porinlike proteins, the 20-kDa protein may also be considered a protective antigen common to both biotypes and serotypes. Recent studies (46) demonstrated that some of the *V. cholerae* strains belonging to classical biotype express a toxin-coregulated pilus (TCP) which has a major protein subunit (TcpA) with a molecular mass of 20 kDa. Although several studies (14, 18) failed to show TCP structures in *V. cholerae* El Tor strains, expression of TcpA antigen could be demonstrated in this biotype as well (18). It is quite possible that the 20-kDa MOMP studied here is also related to TcpA, since expression of this protein could be directly correlated with the production of cholera toxin (41). Furthermore, an antiserum (kindly provided to us by R. H. Hall) raised against *V. cholerae* 0395 (classical, Ogawa) grown under TCP-expressing conditions recognized, after appropriate absorption with the classical strain grown under TCP-repressing conditions (nutrient broth at 37°C) (43), 20-kDa proteins in the OM preparations of *V. cholerae* strains of both classical and El Tor biotypes grown in TSB at 37°C (data not shown). Thus, these findings are consistent with the earlier observation that antisera to TCP can induce protection against challenge with *V. cholerae* organisms belonging to both biotypes (45). Electron microscopic studies carried out by us, however, failed to convincingly demonstrate TCP-like structures in these strains (data not shown). It is possible that the 20-kDa protein is expressed in a membrane-associated nonfimbrial form in these *V. cholerae* O1 cells (18). Interestingly, of several non-O1 strains tested, a cholera toxin-producing strain, V_2, was also found to express antigenically related 20-kDa protein. No information, however, is available in the literature to explain the expression of TcpA-like proteins in non-O1 *V. cholerae*.

Our results demonstrate the involvement of more than one cell surface protein antigen of *V. cholerae* in the induction of protective immunity through inhibition of intestinal colonization of vibrios. It is conceivable that apart from the proteins studied here, other cell surface components of *V. cholerae* expressed in vivo (20) and in vitro (2–4, 8, 12, 19) are involved in protection. Formulation of strategies for effective stimulation of gut-associated immunity in the host through immunization with these antigens should be of considerable help in the development of a vaccine against cholera.

ACKNOWLEDGMENTS

This work was supported by research grant 4/2/89-G from the Department of Atomic Energy, Government of India. Tapas K. Sengupta was the recipient of fellowship 9/15 (39)/88-87 EMR-I from the Council of Scientific and Industrial Research, Government of India.

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