INFECTION AND IMMUNITY, Sept. 1996, p. 3778–3785 Vol. 64, No. 9
0019-9567/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Immune Mechanisms and Protective Antigens of *Vibrio cholerae* Serogroup O139 as a Basis for Vaccine Development

GUNHILD JONSON,† JACEK OSEK,‡ ANN-MARI SVENNERHOLM, AND JAN HOLMGREN*

Department of Medical Microbiology and Immunology, Göteborg University, S-413 46 Göteborg, Sweden

Received 15 August 1995/Returned for modification 1 November 1995/Accepted 20 June 1996

We have characterized 11 isolates of *Vibrio cholerae* O139 Bengal with regard to properties deemed to be relevant for development of a vaccine against O139 cholera. For most strains two colony variants, A and B, which are nonhemolytic and hemolytic, respectively, were detected on blood agar. The A and B variants were associated with high- and low-level production of soluble hemagglutinin-protease, respectively. However, on Luria-Bertani agar both types formed opaque colonies, which has been shown to be associated with capsule formation. Interestingly, under the stationary tube-shaken flask culture conditions in yeast extract-peptone water medium which were used to stimulate the production of cholera toxin (CT) and toxin-coregulated pili, B variants constitutively produced CT and TcpA, two ToxR-regulated proteins, at 28 and 37°C, whereas the production of these proteins by A variants was downregulated at the higher temperature. One of the strains, 4260B, having a well-exposed O antigen and capsule and the capacity to produce large amounts of TcpA, CT, and mannose-sensitive hemagglutinin pili but minimal amounts of the proteolytic soluble hemagglutinin, was selected to produce antibacterial antisera and as a challenge strain in protection studies using the rabbit ileal loop model. Rabbit antisera to live, heat-killed, or formalin-killed O139 vibrios or to purified O139 lipopolysaccharide (LPS) as well as monoclonal antibodies (MAbs) to O139 LPS agglutinated all O139 isolates. However, when A and B variants of strain 4260 were tested for sensitivity to vibriocidal activity of these antibody preparations, only the B variant was killed. All of the antisera against live or killed O139 vibrios conferred passive protection against fluid accumulation induced by the challenge strain. The protective effects of the antisera were correlated to anti-LPS antibody titers rather than to titers against whole bacteria that had been grown for toxin-coregulated pilus expression. This protection was considerably higher than that conferred by antisera to classical, El Tor, or recombinantly produced (classical) CT or CTB. Furthermore, MAbs to O139 LPS and CTB-CT exhibited a strong synergistic protection against O139 challenge irrespective of the level of sensitivity of challenge strains to O139 LPS MAbs in vibriocidal assays in vitro.

The adult population in areas where cholera is endemic in India and southeast Asia is significantly protected against cholera caused by *Vibrio cholerae* of serogroup O1 because of naturally acquired immunity following repeated natural exposure (11, 30). Cholera caused by *V. cholerae* O139 Bengal, a new serogroup that has rapidly spread in southeast Asia after its initial isolation in Madras, India, in 1992, has had a high incidence in both adults and children in areas where cholera is endemic (3), suggesting that there is little if any preexisting immunity against O139 cholera resulting from natural exposure to *V. cholerae* O1.

V. cholerae O139 strains have, with the exception of the O antigen and the production of a capsule, been found to be biochemically and genetically similar to *V. cholerae* O1 of the El Tor biotype (5, 13, 14, 18, 38). O139 vibrios produce cholera toxin (CT) and the structural subunit, TcpA, of toxin-coregulated pilus (TCP) under culture conditions similar to those inducing production of these proteins in El Tor strains, and as in *V. cholerae* O1 strains the production of these two proteins, which are associated with epidemic cholera strains, is dependent on the transcriptional regulator ToxR (38). Furthermore, on the basis of restriction fragment length polymorphism, PCR, or direct sequencing, *ctx* and *tcpA* genes in El Tor and O139 strains appear to be identical, although O139 strains generally have been found to have more copies of the *ctx* genes (13, 24, 27, 38, 39). The major differences between O139 and O1 *V. cholerae* are the composition and lengths of the O-side chains of the cell wall lipopolysaccharide (LPS) and the presence of a capsular polysaccharide (CPS) in O139 strains that is not found in *V. cholerae* O1 strains (6, 15, 18, 31, 40). It has been suggested that the O139 strain arose from an El Tor strain of *V. cholerae* O1 in which a substantial deletion of the *rfb* region occurred, rendering the strain negative with respect to the O1 antigen (4, 6, 31). Instead, it has gained genes responsible for the antigen switch to O139, an event that is suggested to be related to the presence of an insertion site-like sequence within the *rfb* region (4, 6).

The most important protective antigen in *V. cholerae* O139 (as well as in *V. cholerae* O1) is most likely the O antigen of the LPS. The effect of the apparently thin capsule (18), if any, on the exposure of protective antigens, including LPS, has not yet been fully evaluated, and it is not known whether the capsule itself is a protective antigen. Indeed, recent data suggest that the capsule and LPS may have common epitopes and that the capsule is at least partly composed of O-side chain polysaccharides that are not linked to the LPS core (37). Encapsulated O139 vibrios have been found to colonize better than noncapsulated or rough mutants in the infant-mouse model (37), implying that both O139 LPS and the O-antigen capsule are virulence determinants. Wild-type O139 vibrios were also

* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, Göteborg University, Guldhedsgatan 10, S-413 46 Göteborg, Sweden. Phone: (31) 60 49 11. Fax: (31) 82 01 60.

† Deceased, March 1996.

‡ Present address: Department of Microbiology, National Veterinary Research Institute, 24-100 Puławy, Poland.

more resistant to the bactericidal activity of serum in vitro; however, the mechanisms behind the better colonization ability of encapsulated strains are not known (37).

The epidemics of the El Tor-like *V. cholerae* O139 in areas where cholera is endemic suggest that natural exposure, usually asymptomatic, to El Tor-associated antigens per se is not sufficiently protecting against O139 infection and disease. This does not exclude a protective role of, e.g., immunity directed against either CT or the mannose-sensitive hemagglutinin (MSHA) pilus and/or TCP, which are two potential colonization factors and protective antigens in *V. cholerae* El Tor strains (2, 20, 27, 35) that are also produced by *V. cholerae* O139 (22, 35a). Antibodies to MSHA and El Tor TcpA have been found to be protective against *V. cholerae* El Tor infection in animal models (26, 36).

We have examined the expression of candidate protective antigens in a number of *V. cholerae* O139 strains and evaluated the protective capacities of antibacterial and antitoxic antibodies against experimental O139 cholera in the rabbit ileal loop model as a basis for vaccine development including the selection of a candidate vaccine strain. Our results indicate that antibodies to the O-side chain of O139 LPS are strongly protective against challenge with live O139 vibrios, including vibrios with a documented capsule formation and reduced sensitivity to vibriocidal antibodies in vitro. The results also suggest that similar to the situation for immune protection against O1 cholera, antibacterial and antitoxic immune mechanisms are strongly synergistic in protecting against experimental cholera caused by *V. cholerae* O139 even though antitoxic immunity by itself is only marginally effective.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The 11 *V. cholerae* strains of serogroup O139 used in the study are clinical isolates from the early outbreaks of cholera in 1993 in India (Madras) (strains, 2294, 2295, 2297, 2298, 2309, 5520, and 5521) and Bangladesh (International Centre for Diarrhoeal Diseases Research, Bangladesh [ICDDR, B], Dhaka; strains, 1838, 1854, 4260, and 4450). The Indian strains were kindly supplied by M. Tirngad, Madras, India, and the Bangladeshi strains were supplied by M. J. Albert, ICDDR. Strain AI1837, a O139 challenge strain used for human challenge studies at the Center for Vaccine Development, University of Maryland, Baltimore, isolated in Bangladesh in 1993, and used in some comparative experiments, was obtained from M. Levine.

Upon the arrival of the strains in our laboratory on blood agar plates (after a short transport from Stockholm, Sweden), we noted colony variants, which were isolated and stored at −70°C in broth containing 20% (vol/vol) glycerol, for all strains (except strain 1838). Hemolysis around colonies was examined after growth overnight at 37°C on either horse or sheep blood agar plates. Before use, strains were grown on blood agar plates at 37°C for 18 h, and then a short-time culture procedure (21) in yeast extract-peptone water medium (AKI medium: 1.5% Bacto Peptone, 0.4% yeast extract [Difco], 0.5% NaCl, 0.3% $NaHCO_3$) was used in order to avoid proteolytic degradation of any active A subunit of cholera toxin (CTA) produced. Briefly, a 10-ml culture in freshly prepared AKI medium, initially grown in a test tube standing still for 3 h, was transferred to a flask which was incubated with shaking for 2.5 h. These AKI cultures, performed at both 28 and 37°C, were used to determine the production of TcpA-TCP and CT-CTA. In addition, CT production was tested after growth in Syncase (9) overnight and MSHA and soluble hemagglutinin (SHA)-protease production was tested after growth in Trypticase soy broth (TSB) without glucose (BBL, Cockeysville, Md.) at 37°C to exponential and stationary phase, respectively. Luria-Bertani (LB) agar (30) was used to assess the opacity of colonies and in screening for sensitivity to polymyxin B (130 U in biodisks).

Electron microscopy. Bacteria grown overnight on blood agar at 37°C and suspended in phosphate-buffered saline (PBS), pH 7.2, were applied on Formvar-coated 400-mesh nickel grids and incubated for 1 min while being slightly warmed up by a 60-W lamp bulb. After being rinsed three times with water, the grids were placed bacterium side down on 1% (wt/vol) ammonium molybdate, pH 7.0, for 45 s and then dried. Immunoelectron microscopy (IEM) of bacteria incubated with anti-O139 LPS monoclonal antibodies (MAbs) or anti-MSHA MAb HA 17:10 was prepared as described previously (19). Grids were examined in a JEM-1200 EX electron microscope (Jeol Ltd., Tokyo, Japan).

LPS purification. O139 LPS was prepared from strain 4260B. Bacteria were grown overnight in TSB without glucose at 37°C, and crude LPS was prepared essentially by scaling up the method described by Kido et al. (23). Bacteria from a 500-ml culture were suspended in 33 ml of TAE buffer (0.04 M Tris base, 0.002 M EDTA, pH adjusted to 8.5 with acetic acid), mixed with 2 volumes of an alkaline solution (3% [wt/vol] sodium dodecyl sulfate [SDS], 0.05 M Tris base, 0.128 M NaOH), and heated at 60°C for 70 min. After extraction with an equal volume of phenol-chloroform (50:50, vol/vol%), the water phase was obtained after repeated centrifugation at 16,000 × *g* for 10 min and an additional 2/3 volume of H_2O was added. LPS was precipitated by adding 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes of ethanol. After centrifugation at 16,000 × *g* for 20 min, the precipitate was suspended in H_2O and crude LPS (containing slowly migrating [SM], moderately migrating, as well as rapidly migrating [RM] silver-stained material [proteins and RNA]) obtained after discarding undissolved material by centrifugation at 16,000 × *g* for 10 min. Purified O139 LPS was obtained after further treatment with DNase, RNase, and protease, as described earlier (22a). Purified LPS was RNA free (no peak A_{260}), did not contain any protein detected by Coomassie brilliant blue, and was free from SM or moderately migrating material as determined by silver staining following oxidization of samples separated by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (1). O139 LPS was examined for cross-reactivity with O1 Ogawa and Inaba LPSs purified as described above from crude LPS extracted by hot (65°C) phenol-water (50:50, vol/vol%).

Toxin assays and purification. The concentrations of CT in supernatants from short-time AKI and overnight Syncase cultures grown at 28 and 37°C were tested by a GM1 ganglioside–enzyme-linked immunosorbent assay (ELISA) (33) performed in Nunc's low-protein binding polystyrene microtiter plates using a battery of polyclonal antibodies and MAbs against classical and El Tor CT-CTB and -CTA. Cholera toxin from strain 4260B was prepared from a 500-ml AKI short-time culture as described earlier (8) and compared with CT from classical (List Biological Laboratories, Campbell, Pa.) and El Tor *V. cholerae* O1 (8) by SDS-PAGE and immunoblotting of separated proteins by procedures previously described (8, 21). The toxins were also tested in the Chinese hamster ovary (CHO) cell assay (12). The capacities of various anti-CT-CTB antibody preparations to inhibit the biological activity of O139 CT were determined by using CT from classical and El Tor vibrios as references.

SHA-protease. The production of SHA by bacteria grown in TSB without glucose at 37°C overnight was determined by an inhibition ELISA as described previously (34).

TcpA and TCP analysis. Bacteria from short-time AKI cultures that were scored for CT production were analyzed for TcpA by SDS-PAGE and immunoblotting as described previously (19, 21). Samples (4 μl) of suspensions of 10^{10} bacteria ml^{-1} were boiled in the absence of 2-mercaptoethanol. The protein pattern was visualized by Coomassie blue staining and reactivity with rabbit anti-TCP antiserum as well as by immunoblotting with anti-TCP MAb Tc 20:2 (19), recognizing both classical and El Tor TcpA. Surface expression of TCP antigens reactive with anti-TcpA MAb Tc 20:2 was assessed by an inhibition ELISA essentially as described earlier (19), the modification being the use of TCP (classical) (1.5 μg ml^{-1} in 0.05 M carbonate buffer, pH 9.6) as the solid-phase antigen.

MSHA pilus analysis. Bacteria were examined for surface-exposed MSHA pili by hemagglutination of chicken erythrocytes (and inhibition by D-mannose) and by slide agglutination and IEM using anti-MSHA MAb HA 17:10 as described previously (20).

Antisera and MAbs. Adult New Zealand White rabbits (groups of four animals for each antigen) were immunized intramuscularly with purified O139 LPS (100 μg) and live (10^8 bacteria) or heat- or formalin-killed (4×10^9 bacteria) O139 vibrios of strain 4260B grown under the conditions of short-time AKI cultures at 28 to 30°C as described above. Four injections with antigen-bacteria suspended in 1 ml of PBS, without adjuvant, were given at 1-week intervals. One week after the last immunization, animals were bled by heart puncture and sera were obtained and stored at −30°C. Heat- and formalin-inactivated bacteria, respectively, were prepared by incubating bacterial suspensions, 4×10^9 vibrios ml^{-1}, in a shaking water bath at 56°C for 1 h or by adding formaldehyde to a final concentration of 0.2 M and incubating suspensions at room temperature for 24 h and then at 4°C for another 24 h. The vibrios were washed and resuspended in PBS. Complete inactivation was confirmed by testing growth of suspensions on blood agar plates at 37°C for 5 days. Rabbit antiserum against a prototype formalin-inactivated vaccine preparation of *V. cholerae* O139 strain 4260B (bacteria had been grown at 37°C to exponential phase) was obtained from SBL Vaccine, Stockholm, Sweden.

MAbs O139:1:1:9 and O139:20:4:5 against *V. cholerae* O139 LPS were obtained as previously described (7) by immunizing BALB/c mice repeatedly (twice intraperitoneally and once intravenously) with highly purified LPS, 7 μg per dose, prepared from strain 4260B. Specificities of the MAbs were tested by slide agglutination of whole bacteria, ELISA (see below), and SDS-PAGE and immunoblotting (21) of LPS, whole bacteria, and CPS (kindly provided by M. Levine and G. Losonsky, Center for Vaccine Development). The production of MAbs LT 39:1:13 (discriminating between CTB from classical and El Tor *V. cholerae* O1), ETc 31:20 against El Tor CTB, and CT 17:10 against CTA has been described earlier (25).

Serological methods. Antibody titers against O139 LPS, CPS, and whole bacteria (grown in AKI medium at 30°C) were determined by ELISA performed in Nunc's low-protein binding polystyrene microtiter plates as described previously (19). Purified LPS and CPS were applied as a coating to the polystyrene wells at room temperature overnight at concentrations of 2.5 and 5 μg ml^{-1}, respectively,

TABLE 1. Phenotypic characteristics of A and B variants of *V. cholerae* O139[a]

Production of:	Result for colony type[b]	
	A	B
SHA-protease[c] ($\mu g\ ml^{-1}$)	>10	≤0.5
Hemolytic zone on blood agar	No	Yes
CT ($\mu g\ ml^{-1}$) in:		
AKI medium (28°C)	1.2–4.2	4.0–4.2
AKI medium (37°C)	0.8–1.6	4.5–5.4
Syncase (28 or 37°C)	≤0.03	≤0.03
TcpA[d] in:		
AKI medium (28°C)	++	+++
AKI medium (37°C)	(+)	+++
TCP surface antigen[e]	ND	ND
MSHA[f]	+++	+++
LPS on surface[g]	+++	+++
Capsule[h]	Yes	Yes

[a] Based on the examination of 11 strains isolated from Bangladeshi and Indian cholera patients in 1993.

[b] Definitions of results not reported as Yes or No (presence and absence, respectively) or in micrograms per milliliter are as follows: (+), very faint reaction; ++, strong reaction; +++, very strong reaction; ND, not detectable.

[c] As produced in TSB without glucose at 37°C.

[d] Analyzed by SDS-PAGE and immunoblotting.

[e] Analyzed by inhibition ELISA using antibody to classical TCP and classical TCP as solid-phase antigen.

[f] Determined by hemagglutination and reactivity with anti-MSHA MAb HA 17:10.

[g] Determined by agglutination with rabbit anti-O139 LPS and inhibition ELISA.

[h] As indicated by opaque colonies on LB agar (18) and CPS-comigrating antigen on immunoblots and confirmed for selected strains by electron microscopy.

in PBS, and the bacteria, after being washed and resuspended in PBS, were added at a concentration of 5×10^9 organisms ml^{-1}. Antisera were tested in serial threefold dilutions. Titers were determined as the reciprocal dilutions of antibodies giving an A_{450} of 0.4 more than the background level after 20 min. SDS-PAGE and immunoblotting were used for specificity tests.

Vibriocidal antibodies against *V. cholerae* O139 bacteria were determined by a microdilution assay essentially as described previously (17). Bacteria precultured on blood agar were grown in brain heart infusion broth at 37°C and used at a final concentration of 3×10^6 bacteria ml^{-1}. Antisera were inactivated at 56°C for 30 min before use. Because of reduced sensitivity to complement by O139 strains, complement (sterile-filtered guinea pig serum stored in aliquots at −70°C) was used at a final concentration of 7% (vol/vol), i.e., ca. four times the concentration used for El Tor *V. cholerae* O1 strains.

Rabbit ileal loop assay. The capacities of antisera raised against live and heat- and formalin-killed *V. cholerae* O139 vibrios and of antibodies directed against LPS and CT to protect against experimental *V. cholerae* O139 infection were evaluated in passive protection experiments using the rabbit ileal loop test as described previously (26). *V. cholerae* 4260B and AI1837 used for challenge were grown in TSB without glucose with shaking at 37°C for 3 to 4 h, and the challenge dose, 2×10^6 vibrios, corresponded to four times the 50% effective dose ($4\times ED_{50}$) (i.e., four times the dose giving rise to half-maximum fluid accumulation). Antisera were inactivated at 56°C for 30 min, and the dilutions were mixed with equal volumes of bacteria. As a control, bacteria were mixed with PBS. After preincubation for 1 h at room temperature, 1 ml of each antibody-bacterium preparation was injected into at least two randomly positioned 5-cm small intestine loops; each preparation was tested in 2 to 4 rabbits weighing about 1.5 to 2.0 kg. After 16 to 18 h, the animals were sacrificed and the ratio of the volume of accumulated fluid to length of the loop was calculated for each animal.

RESULTS

Phenotypic characterization of *V. cholerae* O139 isolates. We have characterized four Bangladeshi and seven Indian isolates of *V. cholerae* O139 with regard to properties deemed to be relevant for vaccine development against cholera caused by this new serogroup. Most of the isolates presented two colonial types on blood agar, A and B, the B variants producing a small β-hemolytic zone, absent in A types, around each individual colony on either horse or sheep agar plates. More strikingly, A and B variants were associated with high- and low-level production of SHA-protease, respectively (Table 1). High-level production of SHA-protease was correlated with green-colored, clear zones of hemolysis around dense bacterial streaks on blood agar. In addition, a third variant, type C, with smaller and more-elevated colonies, was seen in some isolates. This variant otherwise resembled type B with respect to the phenotypic characteristics listed in Table 1. We were unable to detect any significant difference between types A and B in colony appearance on LB agar. Both types gave rise to opaque colonies, suggesting that capsule material can be produced by both types (18). Indeed, the presence of a capsule was morphologically confirmed by electron microscopy, including IEM using anti-LPS MAbs to stain the cell surface on strain 4260 variants A and B and on strain AI1837 (not shown). Control O1 El Tor strains were translucent and lacked capsule when examined by IEM. All variants were resistant to polymyxin B.

Interestingly, the production of both TcpA and CT, two ToxR-regulated proteins, seemed to be constitutive under AKI culture conditions in B variants; these proteins were produced at equally high levels at 28 and 37°C. At 37°C, A variants, on the other hand, produced less toxin and levels of TcpA that were hardly detectable compared with those expressed at 28°C. (Table 1; also exemplified for strain 4260 in Fig. 1). Furthermore, under these culture conditions at 37°C A variants produced the ToxR-influenced (downregulated by ToxR) outer membrane protein OmpT at an elevated level compared with that at 28°C, whereas B variants did not produce any OmpT as judged by SDS-PAGE (38) (not shown). Surface TCP antigen was not detected by the reagents used (Table 1), although anti-TCP MAb Tc 20:2 reacted with the O139 TcpA subunit in immunoblot analysis (not shown); these results do not exclude, however, the presence of either type-specific or El Tor TCP. A and B variants of all strains produced MSHA, and they all strongly agglutinated chicken erythrocytes in a mannose-sensitive manner. There was no apparent difference in the amounts of LPS produced by A and B variants as tested by slide agglutination using anti-O139 LPS antibodies (polyclonal antibodies or MAbs) or by a quantitative inhibition ELISA using purified O139 LPS as the solid-phase antigen and an antiserum to heat-killed *V. cholerae* O139 bacteria. Also, A and B variant bacteria reacted similarly in SDS-PAGE and immunoblotting with anti-O139 LPS MAbs.

Characterization of candidate protective antigens. CT from strain 4260B was purified and compared with CT from classical and El Tor *V. cholerae* O1. The profiles of A, A1, and B subunits examined by SDS-PAGE and the results from epitope mapping by ELISA using a battery of MAbs allowing for discrimination between classical and El Tor CT (not shown) confirmed that the O139 toxin is structurally and immunologically indistinguishable from El Tor CT, which is consistent with

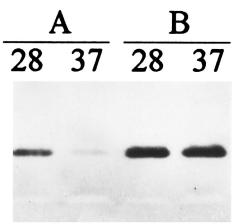

FIG. 1. Immunoblot of SDS-PAGE-separated bacterial lysates of A and B variants of *V. cholerae* O139 strain 4260 showing the differential production of TcpA under short-time AKI culture conditions at 28 and 37°C (lanes 28 and 37, respectively). Transblotted proteins were reacted with rabbit anti-TCP serum (19).

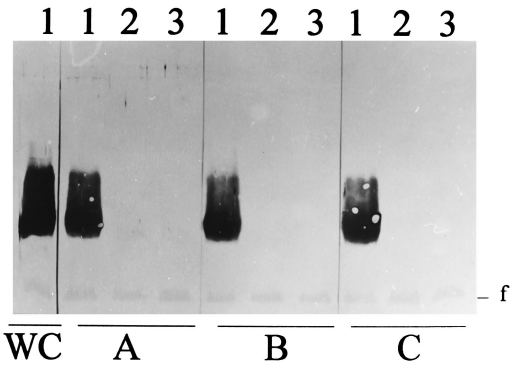

FIG. 2. Purified LPS from *V. cholerae* O139 and O1 separated by SDS-PAGE and immunoblotted with anti-O139 antibodies. Lanes 1, O139 LPS; lanes 2, O1 Inaba LPS; lanes 3, O1 Ogawa LPS. Aliquots (10 μg) of LPS were applied in each well. Blots were probed with rabbit anti-LPS antiserum (A), anti-LPS MAb O139:1:1:9 (B), anti-LPS MAb O139:20:4:5 (C), and rabbit anti-heat-killed O139 (strain 4260B) antiserum (WC). f, front of pyronin Y included in sample buffer.

recently reported DNA sequence data (13, 24). The biological effect in vitro of purified O139 CT was characterized in comparison with El Tor and classical CT by the CHO cell test. The toxins were effective in similar concentrations, and the effect was neutralized by antisera against classical, El Tor, or recombinantly produced (classical) CT or CTB (not shown).

The presence of bacterial surface antigens and potential targets of antibacterial (including vibriocidal) antibodies, including MSHA, TCP, and LPS, was investigated either directly or indirectly via testing of immune sera and MAbs raised against these structures or whole O139 bacteria. All strains were readily agglutinated by anti-MSHA MAb HA 17:10, and it could be demonstrated by IEM that MSHA pili were extending from the bacterial surface. SDS-PAGE comparisons showed that the pili were composed of MshA subunits of identical sizes, ca. 17 kDa, as El Tor MshA. O139 TcpA showed the same reactivity with anti-TCP antibodies in SDS-PAGE and immunoblotting as that previously found for El Tor TcpA (19).

Purified LPS, with the mobility of relatively low-molecular-weight, RM material in SDS-PAGE gels stained with silver, was prepared from strain 4260B, and MAbs and specific polyclonal antisera against O139 LPS were obtained by immunization of rabbits with this material. Such highly purified O139 LPS was also used for immunizing mice and for preparing MAbs specific for LPS. The two MAbs, O139:1:1:9 and O139:20:4:5, which were both of the immunoglobulin G1 isotype, strongly agglutinated A and B variants of all O139 strains but none of the O1 strains tested. In SDS-PAGE and immunoblotting, both the MAbs and polyclonal anti-LPS antibodies reacted with O139 LPS but not with corresponding amounts of Inaba or Ogawa LPS from *V. cholerae* O1 (Fig. 2). However, anti-LPS MAbs (as tested with O139:1:1:9) also reacted in immunoblots of SDS-PAGE gels with higher-molecular-weight, SM material in addition to RM LPS present in O139 bacterial extracts (bacteria grown on LB agar to optimize CPS production) prepared from strain 4260 (A and B types) and which comigrated with SM antigen in CPS from strain AI1837. In addition, the AI1837 CPS also contained some RM LPS antigen. Conversely, purified LPS blotted with antiserum to AI1837 bacteria contained only the RM antigen. Thus, CPS and LPS appear to share at least one strong epitope. This cross-reactivity between RM LPS and SM CPS was further evident in LPS and CPS capture ELISAs (Table 2). The two anti-LPS MAbs reacted in significant titers against both CPS and purified LPS (Fig. 2). However, when the antigen concentration used for coating was reduced to 1 μg ml^{-1}, the MAbs retained their titers against LPS while losing detectable reactivity against CPS. Rabbit anti-LPS antiserum also had a much lower titer against CPS antibody than against LPS (Table 2). While these results might partly be explained by a lower efficiency of coating with LPS in the CPS preparation for the ELISA, they clearly suggest that RM LPS used for immunization has epitopes lacking in CPS.

TABLE 2. ELISA antibody titers and vibriocidal activities of *V. cholerae* O139 rabbit antisera and MAbs used in rabbit ileal loop protection tests

Antibody preparation	ELISA titer[a] against *V. cholerae* O139			Vibriocidal titer[b] against *V. cholerae* O139		
	LPS[c]	WC[d]	CPS[e]	4260A	4260B	AI1837
Rabbit antiserum against:						
Live 4260B WC	35,000	60,000	700	<100	6,400	<100
Heat-killed 4260B WC	118,000	105,000	13,600	<100	12,800	<100
Formalin-killed 4260B WC	47,000	190,000	3,100	<100	6,400	800[f]
Prototype 4260B vaccine[g]	90,000	NT[h]	2,100	<100	12,800	<100
Purified O139 LPS	58,000	35,000	200	<100	6,400	<100
Monoclonal antibodies						
Anti-LPS MAb O139:1:1:9	9,100	NT	3,000[i]	<100	1,600	<100[f]
Anti-LPS MAb O139:20:4:5	3,500	NT	1,300[i]	<10	800	<10

[a] Reciprocal dilutions of antibodies giving an A_{450} of 0.4 more than the background level after 20 min.
[b] Reciprocal dilutions of antibodies causing complete inhibition of bacterial growth in the presence of complement.
[c] Purified low-molecular-weight LPS from strain 4260B.
[d] WC, whole bacteria of strain 4260B grown at 30°C (short-time AKI culture).
[e] CPS, CPS from strain AI1837.
[f] Bacterial killing of <100%. Complement used at 10% (vol/vol); bacteria threefold more diluted than in other experiments.
[g] Formalin-killed 4260B bacteria (grown at 37°C to late exponential phase in TSB without glucose).
[h] NT, not tested.
[i] No titers (<10) were obtained against CPS, whereas titers against LPS remained high, when MAbs were tested in wells coated with antigens at a concentration of 1 μg ml^{-1} (instead of the standard concentrations [for CPS, 5 μg ml^{-1}, and for LPS, 2.5 μg ml^{-1}]).

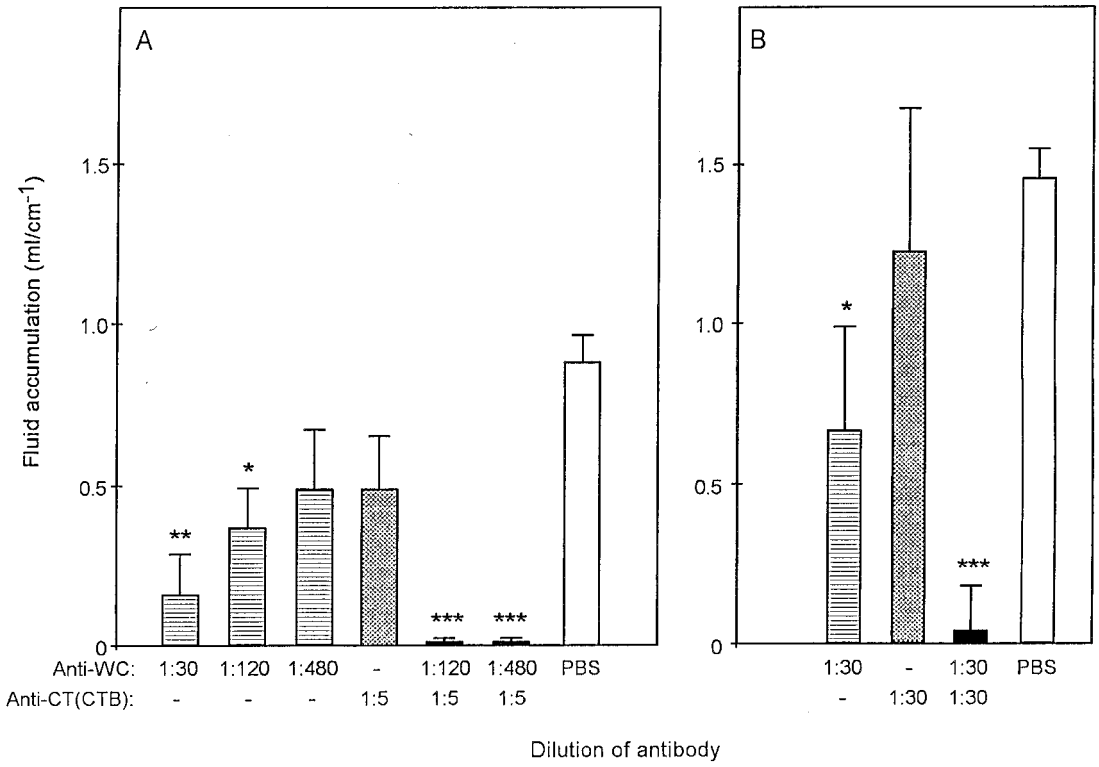

FIG. 3. Synergistic protective effect of rabbit antibacterial and antitoxic antisera against fluid accumulation in rabbit ileal loops caused by challenge with homologous live *V. cholerae* O139 strain 4260B. Shown are the mean values plus standard errors of the means (error bars) of four experiments. (A) Protective effect of anti-heat-killed WC antiserum (Table 2) (striped bars) and anti-CTB antiserum alone (crosshatched bars) and in combination (solid bars). Challenge dose, 2×10^6 bacteria ($4\times$ ED_{50}). (B) Synergistic protective effect of anti-formalin-killed WC antiserum (Table 2) (striped bar) and anti-El Tor CT antiserum alone (crosshatched bar) and in combination (solid bar). Challenge dose, 10^7 bacteria ($20\times$ ED_{50}). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *P* values are relative to the PBS control (open bar).

Antibody titers and vibriocidal activity. Rabbit antisera against O139 LPS and live and heat- or formalin-killed whole bacteria (WC) of strain 4260B were found to agglutinate all O139 strains tested. Their antibody titers against O139 LPS, WC (grown to optimize the expression of TCP-TcpA), and in some cases CPS were determined by ELISA, and their vibriocidal activities against homologous and heterologous strains were assessed (Table 2). The geometric mean titers of antibody against WC, compared with those against LPS, from rabbits immunized with live or formalin-killed vibrios suggest that in addition to containing anti-LPS antibodies these antisera also contained antibodies against non-LPS antigens. When tested against strain 4260B, the vibriocidal antibody titers, however, seemed to correlate with the antibody titers against LPS rather than against WC or CPS. Interestingly, although A and B variants of strain 4260 grown in brain heart infusion medium reacted with equal strengths with the different antibody preparations in slide agglutination tests, the A variant was found to be resistant to the vibriocidal activity of all antibacterial antisera that were vibriocidal against type B bacteria (Table 2). Initially, we had an experience with strain AI1837 similar to that with strain 4260A in the vibriocidal assay. However, when the complement concentration was increased to 10% (vol/vol) and the number of bacteria was reduced to 10^6 bacteria ml^{-1}, the rabbit antiserum tested, but not the anti-O139 LPS MAb, gave a partial vibriocidal effect even though no antibody dilution tested gave rise to 100% killing of the target cells. The two anti-O139 LPS MAbs had detectable vibriocidal activity only against strain 4260B (Table 2).

Virulence and protective immunity. Although the in vitro conditions (temperature) inducing maximum CT and TcpA production differed strikingly for A and B variants, determinations of the number of organisms giving rise to half-maximum fluid accumulation in rabbit intestinal loops (ED_{50}) suggested only a small advantage for the B type (ED_{50}s being 2×10^6 and 0.5×10^6 vibrios for A and B, respectively).

Since strain 4260B produced all candidate protective antigens at high levels, while producing little, if any proteolytic SHA (Table 1), we used this strain both as a prototype immunogen for producing antibacterial antisera and as a prototype challenge strain in passive protection studies in the rabbit small intestine loop model. The challenge dose used, 2×10^6 vibrios per intestinal loop, corresponding to $4\times$ ED_{50}, consistently gave rise to substantial intestinal secretions as tested by several experiments (data not shown).

The protective effects of the rabbit antisera against this challenge, listed in Table 2, were evaluated as were the effects of rabbit antisera against CT and CTB of *V. cholerae* O1 tested both alone and in combination with the antibacterial antisera. All antibacterial antisera tested were found to protect against O139 challenge, and the protective effect was shown to be dependent on the antibody concentration, as exemplified in Fig. 3. Rabbit antiserum against El Tor CTB, on the other hand, provided only partial protection (Fig. 3A). A combination of antibodies against heat-killed O139 vibrios and anti-CTB antibodies, however, resulted in practically complete protection, even when the antibacterial antibodies were diluted as much as 1:480 and used in combination with anti-CTB rabbit

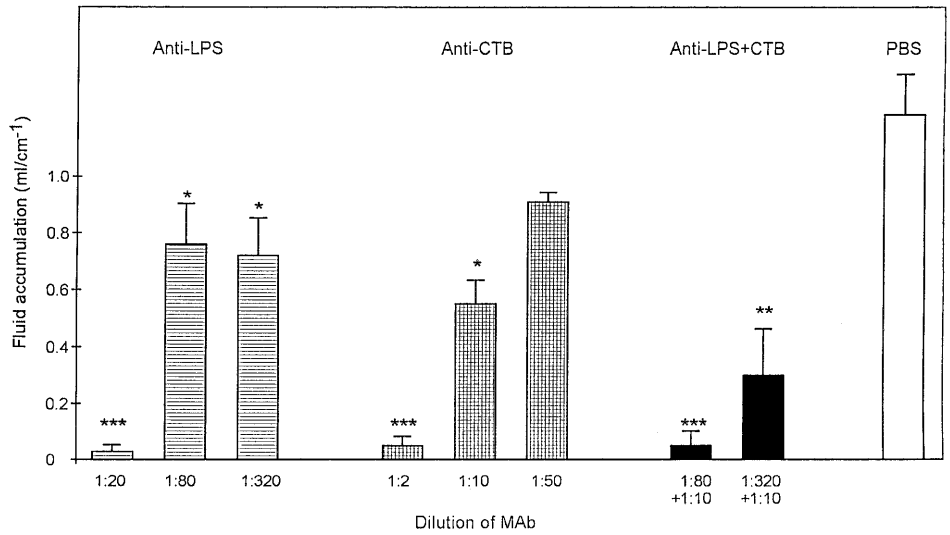

FIG. 4. Synergistic protective effect of MAbs against O139 LPS (MAbs O139:1:1:9 and O139:20:4:5 combined 1:1) (Table 2) and CTB against fluid accumulation in rabbit ileal loops caused by challenge with live *V. cholerae* O139 strain 4260B. Shown are the mean values plus standard errors of the means (error bars) of eight experiments. Challenge dose, 2×10^6 bacteria ($4\times$ ED_{50}). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *P* values are relative to the PBS control (open bar).

antiserum diluted 1:5, concentrations of antisera that repeatedly had been found to be insufficient for significant protection when these antisera were used separately (Fig. 3A). The results show clearly that anti-CTB and antibacterial antibodies cooperate, working synergistically in protection against O139 challenge in the animal model used. Similar synergistic protective effects were obtained when antisera against live (not shown), or formalin-inactivated O139 vibrios (Fig. 3B) were combined with anti-CTB or anti-El Tor CT antibodies.

These results with polyclonal antisera prompted us to evaluate whether MAbs against O139 LPS and *V. cholerae* O1 CTB would protect when used alone and/or in combination. In agreement with the results obtained with polyclonal anti-O139 LPS antiserum (not shown), we found that MAbs against O139 LPS had a protective effect that was dependent on the antibody concentrations used. A strong, practically complete protective effect was achieved when the LPS MAb was diluted 1:20, and MAb against CTB was also protective when used at a high concentration (dilution, 1:2) (Fig. 4). Furthermore, in analogy with the results obtained with polyclonal antisera, we found a strong synergistic protective effect by combining anti-LPS O139 and anti-CTB MAbs; thus, anti-LPS MAb diluted 1:80 or 1:320 and mixed with anti-CTB MAb diluted 1:10, concentrations that repeatedly did not result in protection when the MAbs were used separately, were strongly protective against fluid accumulation caused by *V. cholerae* O139 challenge (Fig. 4).

Parts of the protection experiments were repeated with parallel challenge with strains 4260B and AI1837 (the ED_{50} for the two strains were identical, 0.5×10^6 vibrios). In addition to the previously used antibody preparations, a rabbit antiserum against formalin-killed prototype 4260B O139 WC vaccine (prototype 4260B vibrios grown at 37°C to late exponential phase and then formalin killed) was included (Table 2). The results showed that a similar synergistic protective effect, obtained either by the use of MAbs against O139 LPS and CTB or polyclonal antiserum to 4260B formalin-killed vaccine in combination with anti-CTB MAb, against strains 4260B and AI1837 was achieved. We conclude that antibacterial (mainly anti-LPS) antibodies work synergistically with anti-CTB immunoglobulins in preventing fluid accumulation in rabbit small intestinal loops infected with either of the challenge strains, 4260B or AI1837, of *V. cholerae* O139.

DISCUSSION

The rapid spread of cholera caused by a new serogroup, O139, of *V. cholerae* in India and southeast Asia since 1992 has initiated intense efforts among cholera research laboratories worldwide to characterize virulence determinants or genes in this cholera vibrio as a basis for vaccine development. Our results confirm the results from other groups in that the phenotype of O139 strains, with the exception of the O antigen and capsule, resembles that of the El Tor biotype of *V. cholerae* O1. However, among colony types isolated from patients some interesting and previously not described phenotype variations which are unrelated to capsule formation have been found. We observed two, and for some strains three, colony variants of O139 isolates obtained on blood agar. A green-colored, clear zone of hemolysis in dense bacterial streaks on blood agar, which was associated with the production of SHA-protease, was found in all isolates except one (strain 1838) and was the product of the colony type designated variant A, which lacks the small, clear β-hemolytic zone found around single colonies of B variants. The A and B variants were equally prevalent. Colony variation is not unique for O139 isolates; different colony types, usually designated as opaque or translucent, have been described for O1 as well as O139 strains (10, 18). We have found A and B variants also in El Tor isolates obtained from patients during the early outbreaks of cholera in Peru in 1991. These A and B types were both translucent on LB agar. The existence of A and B variants in *V. cholerae* O1, which are assumed to be nonencapsulated, further suggests that the phenotypic variation described in this work for O139 strains is not correlated with capsule formation. Since B variants of both O1 and O139 appeared as slightly dryish on top of streaks, there may be a connection to *V. cholerae* O1 El Tor variants described as rugose (28).

Similarly to that in *V. cholerae* O1, the expression of virulence genes in O139 strains has been shown to be coordinately

regulated by the transcriptional activator ToxR (38). An interesting observation was that B variants produced high levels of CT and TcpA constitutively under AKI culture conditions, as indicated by the fact that the production of these ToxR-regulated proteins at 37°C was not downregulated compared with that at 28°C. B variants did not produce any OmpT, irrespective of culture temperature, further supporting the argument that ToxR, which is known to downregulate the production of this protein, was active at both temperatures. Our results suggest that at least two O139 vibrio variants can be isolated from patients, one with constitutive production of ToxR-upregulated proteins and another with inducible production of these proteins. This A-B variation, supposedly reflecting capsule production, seems unrelated to the previously described phase variation between opaque and transluscent colonies in O139 and El Tor strains (18).

Another interesting difference between A and B variants of strain 4260 was the difference in susceptibility in vibriocidal assays. Apparently, the 4260A bacteria can escape the action of complement either by destruction or by prohibiting the penetration of complement. The possibility that complement or antibodies can be proteolytically degraded by A variants producing high levels of SHA-protease is less likely, since El Tor vibrios producing high levels of SHA are fully sensitive in the assay used (17). Additionally, strain AI1837, which was less sensitive than 4260B in the assay, appears as a B variant, so the different susceptibilities of strains and variants to the vibriocidal action of complement probably do not correlate specifically with A-B variation. All anti-O139 antisera, and anti-LPS MAbs raised against highly purified LPS devoid of any SM material, were found to agglutinate brain heart infusion medium-grown A and B variants equally well. Our finding that an anti-O139 LPS MAb recognized both contaminating RM LPS and SM antigen, which were absent in our purified LPS preparation, in CPS is in agreement with the results of Waldor et al. (37), suggesting that at least one epitope(s) is shared between LPS O-side chain polysaccharides and CPS. While it is possible that anti-LPS antibody binding to CPS could be less effective than antibody binding to LPS in exerting a vibriocidal action in conjunction with complement, it is likely that binding to either site is sufficient to mediate both bacterial agglutination and in vivo protection. Indeed, the protection against AI1837 achieved by the anti-O139 LPS MAb that could not kill this strain in the vibriocidal assay supports this notion.

Our experiments show that antibacterial antibodies, mainly those to LPS, are highly protective in a dose-dependent way against rabbit internal loop challenge with live homologous or heterologous O139 vibrios. Antitoxic antibodies alone could also protect against live O139 vibrios, but with less efficacy than against *V. cholerae* O1 challenge (32). However, similar to protection against experimental cholera caused by *V. cholerae* O1 bacteria (32) a strong synergistic protective effect was achieved when anti-O139 bacterial antibodies and anti-CTB antibodies were combined, and such synergism was seen irrespective of whether the antibodies were polyclonal or monoclonal and whether the O139 challenge strain was homologous (4260B) or heterologous (AI1837).

Non-LPS bacterial antigens, as exposed on 4260B bacteria grown under TCP-inducing conditions, seemed to be of little if any significance for protection in the animal model used. Antibodies against non-LPS antigens present in the anti-formalin-killed WC serum (Table 2) could not compensate for the comparably low anti-LPS titer in this antiserum; thus, this serum was relatively less protective than other antibacterial antisera even though it had the highest antibody titer against WC of the sera tested. Other animal models based on challenge in nonligated intestines, such as the infant-mouse model, may be used to better evaluate protective anticolonizing activity of antibodies against non-LPS antigens.

On the basis of the data presented herein and additional work supporting the usefulness of the 4260B strain for large-scale vaccine production by fermentation and formalin killing, a second generation B subunit-whole cell cholera vaccine, being bivalent with regard to the WC component (O1/O139), has been prepared in collaboration with SBL Vaccine and is currently being evaluated in phase 1 and phase 2 clinical trials with humans (17a).

ACKNOWLEDGMENTS

We thank M. M. Levine and G. Losonsky at the Center for Vaccine Development for provision of CPS from and antiserum against strain AI1837.

This work was financially supported by grants from the Swedish Medical Research Council (project 16X-3382), the Swedish Agency for Research Cooperation with developing Countries (SAREC), and the World Health Organization.

REFERENCES

1. **Al Hendy, A., P. Toivanen, and M. Skurnik.** 1991. Rapid method for isolation and staining of bacterial lipopolysaccharide. Microbiol. Immunol. **35:**331–333.
2. **Attridge, S. R., E. Voss, and P. A. Manning.** 1993. The role of toxin-coregulated pili in the pathogenesis of *Vibrio cholerae* O1 El Tor. Microb. Pathog. **15:**421–431.
3. **Bhattacharya, M. K., S. K. Bhattacharya, S. Garg, P. K. Saha, D. Dutta, G. B. Nair, B. C. Deb, and K. P. Das.** 1993. Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh. Lancet **341:**1346–1347.
4. **Bik, E. M., A. E. Bunschoten, R. D. Gouw, and F. R. Mooi.** 1995. Genesis of the novel epidemic *Vibrio cholerae* O139 strain: evidence for horizontal transfer of genes involved in polysaccharide synthesis. EMBO J. **14:**209–216.
5. **Calia, K. E., M. Murtagh, M. J. Ferraro, and S. B. Calderwood.** 1994. Comparison of *Vibrio cholerae* O139 with *V. cholerae* O1 classical and El Tor biotypes. Infect. Immun. **62:**1504–1506.
6. **Comstock, L. E., J. A. Johnson, J. M. Michalski, J. G. Morris, and J. B. Kaper.** 1996. Cloning and sequence of a region encoding a surface polysaccharide of *Vibrio cholerae* O139 and characterization of the insertion site in the chromosome of *Vibrio cholerae* O1. Mol. Microbiol. **19:**815–826.
7. **De St. Groth, S. F., and D. J. Scheidegger.** 1980. Production of monoclonal antibodies. Strategy and tactics. J. Immunol. Methods **35:**1–21.
8. **Dubey, R. S., M. Lindblad, and J. Holmgren.** 1990. Purification of El Tor cholera enterotoxins and comparison with classical toxin. J. Gen. Microbiol. **136:**1839–1847.
9. **Finkelstein, R. A., P. Atthasampunna, M. Chulasamaya, and P. Charunmethee.** 1966. Pathogenesis of experimental cholera. Biological activities of purified procholeragen. J. Immunol. **96:**440–449.
10. **Finkelstein, R. A., M. Boesman-Finkelstein, Y. Chang, and C. C. Häse.** 1992. *Vibrio cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. Infect. Immun. **60:**472–478.
11. **Glass, R. I., S. Becker, M. I. Huq, B. J. Stoll, M. U. Khan, M. H. Merson, J. V. Lee, and R. E. Black.** 1982. Endemic cholera in rural Bangladesh, 1966–1980. Am. J. Epidemiol. **116:**959–970.
12. **Guerrant, R. L., L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, and A. G. Gilman.** 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **10:**320–327.
13. **Hall, R. H., F. M. Khambaty, M. H. Kothary, S. P. Keasler, and B. D. Tall.** 1994. *Vibrio cholerae* non-O1 serogroup associated with cholera gravis genetically and physiologically resembles O1 El Tor cholera strains. Infect. Immun. **62:**3859–3863.
14. **Higa, N., Y. Honma, M. J. Albert, and M. Iwanaga.** 1993. Characterization of *Vibrio cholerae* O139 synonym Bengal isolated from patients with cholera-like disease in Bangladesh. Microbiol. Immunol. **37:**971–974.
15. **Hisatsune, K., S. Kondo, Y. Isshiki, T. Iguchi, Y. Kawamata, and T. Shimada.** 1993. O-antigenic lipopolysaccharide of *Vibrio cholerae* O139 Bengal, a new epidemic strain for recent cholera in the Indian subcontinent. Biochem. Biophys. Res. Commun. **196:**1309–1315.
16. **Holmgren, J., J. Osek, and A.-M. Svennerholm.** 1994. Protective oral cholera vaccine based on a combination of cholera toxin B subunit and inactivated cholera vibrios, p. 415–424. *In* K. Wachsmuth, P. A. Blake, and Ø. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. American Society for Microbiology, Washington, D.C.
17. **Jertborn, M., A.-M. Svennerholm, and J. Holmgren.** 1986. Saliva, breast

milk, and serum antibody responses as indirect measures of intestinal immunity after oral cholera vaccination or natural disease. J. Clin. Microbiol. **24:**203–209.

17a. **Jertborn, M., A.-M. Svennerholm, and J. Holmgren.** Intestinal and systemic immune responses in humans after oral immunization with a bivalent B subunit-O1/O139 whole cell cholera vaccine. Vaccine, in press.

18. **Johnson, J. A., C. A. Salles, P. Panigrahi, M. J. Albert, A. C. Wright, R. J. Johnson, and J. G. Morris, Jr.** 1994. *Vibrio cholerae* O139 synonym Bengal is closely related to *Vibrio cholerae* El Tor but has important differences. Infect. Immun. **62:**2108–2110.

19. **Jonson, G., J. Holmgren, and A.-M. Svennerholm.** 1991. Epitope differences in toxin coregulated pili produced by classical and El Tor *Vibrio cholerae* O1. Microb. Pathog. **11:**179–188.

20. **Jonson, G., J. Holmgren, and A.-M. Svennerholm.** 1991. Identification of a mannose-binding pilus on *Vibrio cholerae* El Tor. Microb. Pathog. **11:**433–441.

21. **Jonson, G., J. Holmgren, and A.-M. Svennerholm.** 1992. Analysis of expression of toxin-coregulated pili in classical and El Tor *Vibrio cholerae* O1 in vitro and in vivo. Infect. Immun. **60:**4278–4284.

22. **Jonson, G., M. Lebens, and J. Holmgren.** 1994. Cloning and comparison of the mannose-sensitive hemagglutinin pilin gene in *Vibrio cholerae* serogroup O1 and O139, abstr. BS-21/37. *In* Abstract book of 7th International Congress of Bacteriology and Applied Microbiology Division. Czechoslovak Society for Microbiology, Prague.

22a. **Jonson, G., A.-M. Svennerholm, and J. Holmgren.** 1989. *Vibrio cholerae* expresses cell surface antigens during intestinal infection which are not expressed during in vitro culture. Infect. Immun. **57:**1809–1815.

23. **Kido, N., M. Ohta, and N. Kato.** 1990. Detection of lipopolysaccharide by ethidium bromide staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Bacteriol. **172:**1145–1147.

24. **Lebens, M., and J. Holmgren.** 1994. Structure and arrangement of the cholera toxin genes in *Vibrio cholerae* O139. FEMS Microbiol. Lett. **117:**197–202.

25. **Lindholm, L., J. Holmgren, M. Wikström, U. Karlsson, K. Andersson, and N. Lycke.** 1983. Monoclonal antibodies to cholera toxin with special reference to cross-reactions with *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **40:**570–576.

26. **Osek, J., A.-M. Svennerholm, and J. Holmgren.** 1992. Protection against *Vibrio cholerae* El Tor infection by specific antibodies against mannose-binding hemagglutinin pili. Infect. Immun. **60:**4961–4964.

27. **Rhine, J. A., and R. K. Taylor.** 1994. TcpA pilin sequences and colonization requirements for O1 and O139 *Vibrio cholerae*. Mol. Microbiol. **13:**1013–1020.

28. **Rice, E. W. C., J. Johnson, R. M. Clark, K. R. Fox, D. J. Reasoner, M. E. Dunnigan, P. Panigrahi, J. A. Johnson, and J. G. Morris, Jr.** 1992. Chlorine and survival of "rugose" *Vibrio cholerae*. Lancet **340:**740.

29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

30. **Siddique, A. K., K. Zaman, A. H. Baqui, K. Akram, P. Mutsuddy, A. Eusof, K. Haider, S. Islam, and R. B. Sack.** 1992. Cholera epidemics in Bangladesh: 1985–1991. J. Diarrhoeal Dis. Res. **10:**79–86.

31. **Stroeher, U. H., K. E. Jedani, B. K. Dredge, R. Morona, M. H. Brown, L. E. Karageorgos, M. J. Albert, and P. A. Manning.** 1995. Genetic rearrangements in the rfb regions of *Vibrio cholerae* O1 and O139. Proc. Natl. Acad. Sci. USA **92:**10374–10378.

32. **Svennerholm, A.-M., and J. Holmgren.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun. **13:**735–740.

33. **Svennerholm, A.-M., and J. Holmgren.** 1978. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM1-ELISA) procedure. Curr. Microbiol. **1:**19–23.

34. **Svennerholm, A.-M., G. Jonson Strömberg, and J. Holmgren.** 1983. Purification of *Vibrio cholerae* soluble hemagglutinin and development of enzyme-linked immunosorbent assays for antigen and antibody quantitations. Infect. Immun. **41:**237–243.

35. **Voss, E., and S. R. Attridge.** 1993. In vitro production of toxin-coregulated pili by *Vibrio cholerae* El Tor. Microb. Pathog. **15:**255–268.

35a. **Voss, E., and S. R. Attridge.** Personal communication.

36. **Voss, E., P. A. Manning, and S. A. Attridge.** Submitted for publication.

37. **Waldor, M. K., R. Colwell, and J. J. Mekalanos.** 1994. The *Vibrio cholerae* O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants. Proc. Natl. Acad. Sci. USA **91:**11388–11392.

38. **Waldor, M. K., and J. J. Mekalanos.** 1994. ToxR regulates virulence gene expression in non-O1 strains of *Vibrio cholerae* that cause epidemic cholera. Infect. Immun. **62:**72–78.

39. **Waldor, M. K., and J. J. Mekalanos.** 1994. Emergence of a new cholera pandemic: molecular analysis of virulence determinants in *Vibrio cholerae* O139 and development of a live vaccine prototype. J. Infect. Dis. **70:**278–283.

40. **Weintraub, A. G., P.-E. Widmalm, M. Jansson, K. Hultenby, and M. J. Albert.** 1994. *Vibrio cholerae* O139 Bengal possesses a capsular polysaccharide which may confer increased virulence. Microb. Pathog. **16:**235–241.

Editor: J. R. McGhee