INFECTION AND IMMUNITY, July 1994, p. 2901–2907
0019-9567/94/$04.00+0

Vol. 62, No. 7

Role of Antibodies against Biotype-Specific *Vibrio cholerae* Pili in Protection against Experimental Classical and El Tor Cholera

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

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

Received 29 December 1993/Returned for modification 9 March 1994/Accepted 15 April 1994

***Vibrio cholerae* O1, which exists as two biotypes, classical and El Tor, expresses fimbrial antigens called toxin-coregulated pili (TCP) and mannose-sensitive hemagglutinin (MSHA) pili, respectively. We have raised rabbit antisera and monoclonal antibodies against these fimbrial antigens and prepared Fab fragments which possess specific antibodies directed against the respective fimbrial antigens from these antisera. The protective effect of these antibody preparations was studied in the infant mouse cholera model. Antibodies against TCP were able to protect baby mice against challenge with *V. cholerae* O1 of the classical but not of the El Tor biotype. Similar but reverse biotype differences in protection against challenge with classical and El Tor vibrios were observed when antibodies against MSHA pili were used. The protective effect of *V. cholerae* O1 antilipopolysaccharide (anti-LPS) antibodies, both alone and in combination with antifimbrial antibodies, was also evaluated. We showed that antibodies to the LPS component also prevented infections with *V. cholerae* O1. Moreover, our results indicate that antibodies against TCP or MSHA pili and against LPS cooperate at least additively, and possible even synergistically, in protecting baby mice against challenge with group O1 vibrios. These results indicate that TCP and MSHA pili as well as LPS play an important role in the pathogenesis of experimental cholera. We could also demonstrate that antibacterial immunity preventing colonization is biotype specific. Our results might be used for the generation of new oral cholera vaccines including both TCP and MSHA fimbrial antigens.**

Vibrio cholerae of O group 1 is the main causative agent of epidemic cholera. *V. cholerae* O1 exists as two biotypes, classical and El Tor, and as two main serotypes, Inaba and Ogawa. Cholera disease is caused by the action of cholera toxin, which gives rise to electrolyte secretion and fluid loss from the small intestine through its stimulation of adenyl cyclase activity (13). However, colonization of the small intestine by *V. cholerae* bacteria is an early important step in the pathogenesis of diarrhea. Different putative adhesive factors have been proposed to be involved in colonization (7, 9, 11, 15, 16, 20, 23). One of these, a toxin-coregulated pilus (TCP), has been shown to be important for colonization by *V. cholerae* O1 of the classical biotype (12, 30, 32, 37). The expression of TCP on the bacterial surface as well as the importance of this pilus as a colonization factor of vibrios of the El Tor biotype, on the other hand, has been questioned (9, 18, 23, 30, 33). Jonson et al. (19) have demonstrated that most or all strains of *V. cholerae* of the El Tor biotype instead express another pilus structure called the mannose-sensitive hemagglutinin (MSHA) pilus. We have also demonstrated that MSHA pili appear to play an important role in the pathogenesis of cholera caused by the El Tor biotype of *V. cholerae* O1 and that specific antibodies against MSHA pili were able to protect against experimental cholera caused by El Tor vibrios (25).

Studies have shown that the protective mechanism against cholera is directed against both bacteria and toxin (14, 26, 34, 35). The antibacterial response is mainly against the lipopolysaccharide (LPS) but also against other bacterial components, e.g., outer membrane proteins and pili (2, 14, 17, 28, 29, 31). Bacterial pili are generally good immunogens for eliciting a local immune response, with the potential to prevent infection by blocking intestinal colonization by *V. cholerae* strains. Thus, an immune response directed against *V. cholerae* pili, such as TCP and MSHA pili, that function in the colonization process could potentially block the infection. We earlier demonstrated that antibodies against MSHA pili could protect against experimental El Tor cholera, probably by inhibiting intestinal colonization (25).

The aim of this study was to extend this observation as well as to evaluate the capacity of antibodies against TCP and MSHA pili to protect against experimental classical as well as El Tor cholera in the infant mouse model. We have also evaluated the capacity of antifimbrial antibodies to cooperate synergistically with anti-LPS antibody preparations for protection against infection with O1 vibrios by determining the protective effect of antibodies directed against these surface antigens, both alone and in combination with anti-LPS immunoglobulins (Igs), against intestinal challenge with vibrios of the classical and El Tor biotypes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Two *Vibrio cholerae* O1 classical strains (395 [Ogawa] and X28214 [Inaba]) and two El Tor strains (174 and T19479, both Inaba serotype) were used for the infant mouse passive protection study. Strain JS1569, a rifampin-resistant derivative of strain CVD 103 (strain 569B *ctxA*; J. Kaper, Baltimore, Md.), was used for the production of antibodies against TCP. Strain 569B/165, a conjugate between *V. cholerae* 569B and the non-O1 serogroup vibrio strain 165 (S. R. Attridge, Adelaide, Australia), was used for purification of TCP. All the strains were stored at −70°C in broth containing 20% (vol/vol) glycerol.

For TCP expression, bacteria were grown by the AKI-SW method (15) at 30°C as described previously (18); these conditions were shown to lead to high levels of TCP (or TcpA, the structural subunit of TCP) expression (20). AKI medium

* Corresponding author. Present address: Department of Microbiology, National Veterinary Research Institute, 24-100 Pulawy, Poland. Fax: (48) 831 2595.

was freshly prepared and adjusted to pH 7.2 to 7.4 by adding sterile $NaHCO_3$ (0.018 M, final concentration) immediately prior to use.

For MSHA pilus expression, cultures were made in Trypticase soy broth without glucose (BBL Microbiology Systems, Cockeysville, Md.) at 37°C for 4 to 6 h with shaking (200 rpm), conditions shown to lead to high-level expression of MSHA fimbriae (21).

Purification of TCP. TCP was purified essentially by a method described previously (10). Briefly, bacteria were grown under the AKI-SW conditions for 16 h at 30°C, harvested by centrifugation (10,000 × *g*, 20 min, 4°C), and washed once with phosphate-buffered saline, pH 7.2 (PBS). TCP fimbriae were extracted from the cell surface by washing with sucrose (152 mM final concentration) and precipitated overnight with salts (6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 2.7 mM KCl, and 0.8 mM NaN_3). The precipitate was centrifuged at 25,000 × *g* for 45 min at 4°C, and the pellet was resuspended in sucrose (152 mM) and centrifuged again at 45,000 × *g* for 45 min at 4°C. The final pellet containing TCP fimbriae was resuspended in sucrose (152 mM) and stored frozen at −70°C.

Purification of MSHA pili. MSHA pili were purified from El Tor strain 174. Bacteria were grown to the late logarithmic phase in Trypticase soy broth without glucose, harvested by centrifugation (10,000 × *g*, 20 min, 4°C), and washed once with PBS. Crude MSHA pili were isolated from the cell surfaces by shearing bacteria five times for 1 min each on ice with an Ultra-Turrax T25 (Janke & Kunkel, IKA-Labortechnik, Staufen, Germany). After centrifugation (12,000 × *g*, 20 min, 4°C), the supernatant containing MSHA fimbriae, flagella, and other membrane vesicles was centrifuged again at 85,000 × *g* for 1 h at 4°C to pellet flagella and membranes. MSHA pili were precipitated from the supernatant by ammonium sulfate precipitation (50% saturation) and dialyzed against 0.05 M Tris-HCl buffer with 0.002 M EDTA, pH 7.5. Further purification was performed on a cesium chloride ultracentrifugation gradient (1.31 g of CsCl per ml) at 110,000 × *g* for 20 h at 15°C. After dialysis against 0.05 M Tris-HCl buffer (pH 7.5), the purified MSHA pili were stored at −70°C.

Purification of LPS. Purified LPS from *V. cholerae* Inaba and Ogawa strains was prepared from strains 569B and 34, respectively, by hot phenol-water extraction followed by repeated ultracentrifugations (24). The preparations were further treated in sequence with DNase, RNase, and protease (Sigma Chemical Co., St. Louis, Mo.), each enzyme being added at 0.01 mg/mg of crude LPS. LPS materials were finally boiled for 10 min, pelleted, and washed twice by repeated ultracentrifugation (105,000 × *g* for 2 h each). The absence of RNA was confirmed spectrophotometrically (A_{260}), and the protein content was less than 0.5%, as determined by the method of Bradford (4).

Antisera and MAbs. An antiserum to TCP was prepared in adult New Zealand rabbits immunized with classical strain JS1569 grown by the AKI method. The initial two injections (10^9 bacteria in each) were given subcutaneously with Freund's complete adjuvant and were followed by a number of intravenous injections at 1-week intervals. The rabbits were bled 1 to 2 weeks after the last immunization, and sera were collected and absorbed twice with boiled and twice with live serotype-homologous, TCP-negative bacteria (18).

An antiserum to MSHA pili was prepared in rabbits given three to five subcutaneous injections of crude MSHA pili (500 μg of protein per ml) at 2-week intervals. The initial two injections were given in Freund's complete adjuvant. The hyperimmune serum was extensively absorbed with classical, MSHA pilus-negative bacteria of the homologous serotype (36).

Antisera against purified LPS of the Inaba and Ogawa serotypes were prepared by immunization of rabbits with 1.25 mg of the LPS preparations, given three to five times subcutaneously; the initial two injections were given in Freund's complete adjuvant.

Monoclonal antibodies (MAbs) against TCP and MSHA pili were produced essentially as described before (6) by immunizing BALB/c mice repeatedly with crude TCP and MSHA pili, respectively. The MAbs against TCP, TCP 20:2 and TCP 21:1, were of the IgG1 isotype and reacted with both purified TCP in an enzyme-linked immunosorbent assay (ELISA) and with TcpA, the structural subunit of TCP, in immunoblot analyses (see below). Ascites fluids containing 23.1 mg of Ig (MAb TCP 20:2) and 30 mg of Ig (MAb TCP 21:1) per ml were obtained by inoculating the hybrid cells into the peritoneal cavities of mice (19).

A MAb to MSHA pili, MSHA 17:10 (IgG3 isotype), produced in mice, reacted with purified MSHA pili and with whole El Tor vibrios by both ELISA and immunoblot but not with LPS or MSHA pilus-negative classical vibrios (19). Electron microscopy studies have shown that this MAb binds along the entire MSHA fimbrial structure (18). Ascites fluid contained 27 mg of Ig per ml.

Preparation of Fab fragments of Igs. Fab fragments of rabbit antibodies against TCP, MSHA pili, LPS Inaba, and LPS Ogawa were prepared essentially as described before (27). The Igs were precipitated with ammonium sulfate (50% saturation), and after centrifugation (13,000 × *g* for 20 min), the pellet was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, and dialyzed against the same buffer for 24 h. Fab fragments of Igs were digested by papain attached to agarose beads (Sigma; final concentration, 1.25 mU/ml) suspended in 0.1 M phosphate buffer with cysteine-HCl (10 mM, final concentration) and EDTA (2 mM final concentration) at 37°C for 16 h. After centrifugation, the supernatant was filtered through a G25M Sephadex PD10 column (Pharmacia, Uppsala, Sweden). The Fab fragments of Igs were tested for the presence of specific antibodies by ELISA and immunoblot analyses.

ELISA. The specificity of Fab fragments of polyclonal antisera as well as the whole Igs of the MAbs (and, for control purposes, preimmune sera and nonspecific MAb) against TCP, MSHA pili, LPS Inaba, and LPS Ogawa has been amply documented by both functional and morphological criteria by ELISA. Polystyrene microtiter plates (Dynatech, Chantilly, Va.) were coated with purified antigens (ca. 1 μg/ml) diluted in PBS (MSHA pili, LPS Inaba, and LPS Ogawa) or in 0.05 M carbonate buffer, pH 9.6 (TCP), at room temperature overnight. After the plates were washed with PBS, any remaining binding sites were blocked with 1% bovine serum albumin (BSA; Sigma) in PBS for 30 min at 37°C. Antibody preparations were serially diluted in the plates, and goat anti-mouse or goat anti-rabbit Ig conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, Pa.) was used as the secondary antibody. Titers are expressed as the reciprocal of the dilutions giving an A_{450} of 0.4 above the background.

Immunoblotting. TCP, MSHA pili, LPS Inaba, and LPS Ogawa preparations were boiled in sample buffer containing sodium dodecyl sulfate (SDS) and 2-mercaptoethanol, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 17% polyacrylamide minigels, and electroblotted to nitrocellulose paper essentially as described previously (18, 19). After being blocked with 1% BSA in PBS, the nitrocellulose sheets were incubated for 2 h with different antibody prepara-

TABLE 1. ELISA titers of antibody preparations used in the infant mouse cholera model

Antibody	ELISA titer[a] against:			
	TCP	MSHA pili	LPS Inaba	LPS Ogawa
TCP antiserum	79,400	NT[b]	7,400	200
Fab fragments of TCP antiserum	2,700	NT	300	20
Preimmune serum	70	NT	<50	NT
TCP 20:2 MAb	>218,000	NT	<30	<30
TCP 21:1 MAb	>218,000	NT	<30	<30
Nonspecific MAb	<30	NT	<30	<30
MSHA pilus antiserum	NT	107,000	400	NT
Fab fragments of MSHA pilus antiserum	NT	19,000	NT	NT
Preimmune serum	NT	150	<30	NT
MSHA pilus 17:10 MAb	NT	42,600	<30	<30
Nonspecific MAb	NT	<30	<30	<30
Fab fragments of LPS Inaba antiserum	<10	NT	12,500	4,700
Fab fragments of LPS Ogawa antiserum	<10	NT	2,100	3,300

[a] Titers were determined as the interpolated reciprocal dilutions giving an absorbance of 0.4 above the background.
[b] NT, not tested.

tions and subsequently for 2 h with peroxidase-labeled goat anti-rabbit or goat anti-mouse IgG (Jackson). Strips were washed extensively with PBS–0.05% Tween (three times) and finally with PBS without Tween before the development step. H_2O_2 with 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, Calif.) was used as the enzyme substrate. Transblotted low-molecular-weight reference proteins (Bio-Rad) were stained for 5 min with 0.1% amido black.

Infant mouse test. The infant mouse cholera model was used to determine the protective effect of different antibody preparations in vivo (3, 25). Bacteria were grown under either TCP- or MSHA pilus-expressing conditions as described above. The bacterial cells were harvested by centrifugation (8,000 × *g*, 10 min, 4°C), washed with PBS, adjusted to a concentration of 10^{10} vibrios per ml (according to the optical density at 600 nm), centrifuged (13,000 × *g* for 5 min), and resuspended to the same volume with the respective antibody preparation or, for control purposes, with a nonspecific MAb or preimmune serum. The bacterium-antibody mixtures were incubated for 1 h at room temperature before being administered to the mice. Three- to 5-day-old Swiss mice that had been separated from their mothers and starved for 4 to 5 h before the challenge were used for the test. Each mouse was inoculated orally with a 50-μl volume (5×10^8 vibrios per mouse) with an eye needle (Unimed, Lausanne, Switzerland) and a syringe. Groups of 5 to 16 mice were given each bacterium-antibody mixture or bacterium-preimmune serum (or nonspecific MAb). Thereafter, the mice were inspected several times daily for diarrhea and death. The protective effect of specific antibodies was determined by comparing the survival of mice given specific anti-TCP, anti-MSHA pili, or anti-LPS antibodies or a combination of these with that of mice given the same bacterial challenge and nonspecific antibodies (or preimmune serum) after 48 h. Protective efficacy (PE) was calculated as follows: [1 − (rate ratio of dead mice given specific antibodies/rate ratio of dead mice given nonspecific antibodies)] × 100 (5).

Statistical analyses. Statistical analyses were performed by Fisher's exact test.

RESULTS

Specificity of antibodies. The specificity of the antibodies against TCP, MSHA pili, and LPS (Inaba and Ogawa) used for protection studies has been documented by ELISA and immunoblot tests (Table 1 and Fig. 1). The antiserum against TCP (which was absorbed with TCP-negative bacteria of the homologous serotype) but not preimmune serum reacted with purified TCP antigen in both ELISA and immunoblot analyses. However, some probably unspecific reactivity with the purified LPS Inaba antigen was also observed (Table 1). Both anti-TCP MAbs used in the study reacted at high ELISA titers with the TCP antigen but not with LPS (Table 1).

The different antibody preparations used in the infant mouse cholera model varied in their reactivities with the TcpA subunit of El Tor and classical vibrios, as tested in immunoblot analyses; the polyclonal antiserum reacted with equal strength with both classical and El Tor TcpA, and the MAb TCP 20:2 also reacted well with both; MAb TCP 21:1, on the other hand, did not react with TcpA of El Tor vibrios and thus seems to be classical biotype specific (data not shown).

The rabbit antiserum against MSHA pili contained antibod-

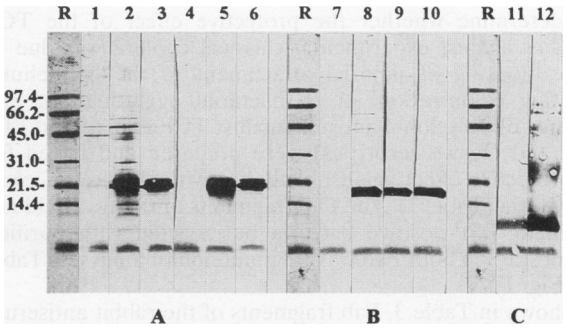

FIG. 1. Immunoblot analysis showing the specificity of the antibodies used in the infant mouse cholera test. Purified TCP (A), MSHA pilus (B), and LPS Inaba and LPS Ogawa (C) antigens were separated by SDS-PAGE, transblotted, and developed with different antibody preparations: lane 1, TCP preimmune serum; lane 2, TCP antiserum; lane 3, Fab fragments of TCP antiserum; lane 4, nonspecific MAb; lane 5, TCP 20:2 MAb; lane 6, TCP 21:1 MAb; lane 7, MSHA pilus preimmune serum; lane 8, MSHA pilus antiserum; lane 9, Fab fragments of MSHA pilus antiserum; lane 10, MSHA pilus 17:10 MAb; lane 11, Fab fragments of LPS Inaba antiserum; lane 12, Fab fragments of LPS Ogawa antiserum. Lanes R, molecular mass markers in kilodaltons (amido black stained). All antibody preparations were tested at comparable Ig concentrations: sera and MAbs, 1:2,000; Fab fragments of TCP, MSHA pilus, and LPS Inaba antisera, 1:500; and Fab fragments of LPS Ogawa antiserum, 1:200.

TABLE 2. Protective effect of anti-TCP antibodies against experimental infection with classical but not with El Tor *V. cholerae* strains in the infant mouse cholera model

Antibody prepn (dilution used)	No. of survivors/total[a] (PE, %)			
	Classical		El Tor	
	395	X28214	174	T19479
TCP antiserum (1:30)	15/15 (100)***	13/15 (67)*	3/15 (0)	4/15 (8)§
Preimmune serum (1:30)	3/15	4/10	0/10	2/10
TCP 20:2 MAb (1:30)	6/7 (78)*	7/9 (78)*	0/9 (0)	0/9 (0)
TCP 21:1 MAb (1:30)	5/7 (55)§	8/9 (83)*	0/9 (0)	0/9 (0)
Nonspecific MAb (1:30)	5/14	3/9	0/9	1/9

[a] ***, $P < 0.001$; *, $P < 0.05$; §, not significant ($P = 0.1$), all versus preimmune serum or nonspecific MAb (Fisher's exact test).

ies directed mainly against MSHA pili (a very low titer against LPS was also evident in the ELISA); MSHA pilus MAb 17:10 reacted only with the homologous antigen and not at all with LPS in both ELISA and immunoblot analyses.

Antibodies against purified LPS (Inaba and Ogawa serotypes) were used as Fab fragments of Igs and did react with the purified LPS antigens in both ELISA and immunoblot tests (Table 1 and Fig. 1).

Protective effect of TCP and LPS antibodies. The capacity of the anti-TCP antibodies to protect against experimental infection with classical and El Tor *V. cholerae* strains was evaluated in the infant mouse cholera model (Table 2). Two strains of each biotype and the whole-antibody Igs were used. The results indicate that antibodies against TCP have a protective effect against challenge with *V. cholerae* of the classical but not of the El Tor biotype. Protection against both of the classical strains was observed with both rabbit polyclonal antiserum and the two different anti-TCP MAbs. The protective effect was significantly superior to that obtained with preimmune serum or a nonspecific MAb of the same Ig concentration. In contrast, no significant protective effect was observed when the mice were challenged with bacteria of the El Tor biotype mixed together with the specific anti-TCP preparations (rabbit antiserum or MAbs).

To determine whether the protective effect of the TCP antibodies against experimental classical cholera was due to specific blocking of fimbrial attachment to the epithelium, preventing colonization, or to bacterial agglutination, Fab fragments of polyclonal antisera against TCP and against LPS (Inaba and Ogawa serotypes) were prepared and tested for their protective effect against challenge with classical *V. cholerae* bacteria (Table 3). The Fab fragments of the Igs could not agglutinate TCP-positive bacteria but reacted with purified TCP and LPS in both ELISA and immunoblot analyses (Table 1 and Fig. 1).

As shown in Table 3, Fab fragments of the rabbit antiserum against TCP were found to give significant protection against challenge with the classical *V. cholerae* strains 395 and X28214. The protective effect of the TCP antibodies depended on the concentration of the Fab fragments of the specific Igs.

To determine whether anti-LPS antibodies also have a protective effect against experimental classical cholera, the Fab fragments of the rabbit polyclonal antiserum against LPS Inaba and against LPS Ogawa were used in the infant mouse test. These antibody preparations were also found to have a significant protective effect against challenge with both classical *V. cholerae* strains used (Table 3).

Since both anti-TCP and anti-LPS antibodies gave protection against classical cholera, a possible synergistic protective effect of these antibody preparations was evaluated. Infant mice were challenged with two classical *V. cholerae* strains, 395 (Ogawa) and X28214 (Inaba), that had been incubated with a mixture of TCP and LPS (Ogawa and Inaba) Fab fragments of the rabbit antibodies. The concentration of each antibody preparation alone used in the test had in previous experiments been found to be too low for significant protection of baby mice against challenge with the classical *V. cholerae* strains. However, as shown in Table 3, the combination of both antibody preparations (anti-TCP and anti-LPS) resulted in markedly stronger protection against *V. cholerae* challenge than the protection achieved with each Fab preparation alone; in fact, the combination had a PE value higher than the sum of the PEs of the two antisera used separately, consistent with at least an additive effect and possibly a truly synergistic cooperative protective effect.

Protective effect of MSHA pilus and LPS antibodies. The capacity of anti-MSHA pilus antibodies to protect against experimental infection with El Tor and classical *V. cholerae* strains was also evaluated with the infant mouse cholera model (Table 4). One strain of each biotype was used, strain 174 of the El Tor and strain X28214 of the classical biotype, and the protective effect of the whole Igs was evaluated. The results

TABLE 3. Protective effects of Fab fragments of anti-TCP and anti-LPS antibodies, both alone and in combination, against experimental infection with classical *V. cholerae* strains in the infant mouse cholera model

Antibody prepn and dilution	No. of survivors/total[a] (PE, %)	
	395	X28214
TCP antiserum		
1:5	7/10 (65)**	4/5 (75)*
1:10	6/10 (54)*	3/5 (50)§
1:15	2/10 (8)§	3/10 (13)§
1:20	0/5 (0)§	1/5 (0)§
LPS antiserum[b]		
1:10	7/10 (65)**	5/5 (100)**
1:20	6/10 (54)*	5/10 (38)§
1:25	4/10 (31)§	2/5 (25)§
1:50	1/10 (0)§	0/5 (0)§
Preimmune sera (1:30)	2/15	2/10
TCP antiserum (1:15) plus LPS Ogawa antiserum (1:25)	8/10 (77)**	NT[c]
TCP antiserum (1:15) plus LPS Inaba antiserum (1:20)	NT	9/10 (88)**

[a] **, $P < 0.01$; *, $P < 0.05$; §, not significant ($P = 0.1$), all versus preimmune sera (Fisher's exact test).

[b] LPS Ogawa antiserum was used for strain 395, and LPS Inaba antiserum was used for strain X28214.

[c] NT, not tested.

TABLE 4. Protective effect of anti-MSHA pilus antibodies against experimental infection with El Tor but not with classical *V. cholerae* strains in the infant mouse cholera model

Antibody prepn (dilution)	No. of survivors/total[a] (PE, %)	
	174 (El Tor)	X28214 (classical)
MSHA pilus antiserum (1:50)	10/12 (76)**	2/13 (15)§
Preimmune serum (1:50)	5/16	0/12
MSHA pilus MAb (1:30)	8/12 (64)**	1/12 (0)§
Nonspecific MAb (1:30)	1/12	2/13

[a] **, $P < 0.01$; §, not significant ($P > 0.25$), both versus preimmune serum or nonspecific MAb (Fisher's exact test).

indicate that antibodies against MSHA pili have a significant protective effect against challenge with *V. cholerae* O1 bacteria of the El Tor but not of the classical biotype. Protection against El Tor strain 174 was observed with both absorbed polyclonal rabbit antiserum and the MAb (Table 4); the effect was superior to that obtained with preimmune serum or a MAb of irrelevant specificity. The significant protective effect of the MSHA pilus antibody preparations was observed even when the antiserum and MAb were diluted as much as 1:50 and 1:30, respectively.

To evaluate whether the protective effect of the anti-MSHA pilus antibodies was due to interference of the specific Igs with binding of the *V. cholerae* bacteria to mucosal receptor structures or to agglutination of the bacteria in the intestinal lumen, and to test a possible protective effect of antibodies to purified *V. cholerae* LPS, Fab fragments of the polyclonal antibodies were evaluated in the infant mouse cholera model (Table 5). The Fab fragments of the MSHA pilus and the LPS (Inaba and Ogawa) antibodies used in the study did not agglutinate MSHA pilus-positive bacteria but reacted strongly with purified MSHA or LPS antigens, respectively, both in immunoblot (Fig. 1) and in ELISA (Table 1) analyses. When tested at an Ig concentration corresponding to that of the MSHA pilus antiserum (Fab fragment preparation diluted 1:12; Table 5), the Fab fragments were also found to offer significant protection against challenge with two El Tor *V. cholerae* strains, 174 and T19479 (PEs of 71 and 57%, respectively, of that obtained with preimmune serum). As shown in Table 5, the protective effect of the MSHA pilus antibodies was proportional to the specific Ig concentration.

TABLE 5. Protective effects of Fab fragments of anti-MSHA pilus and anti-LPS antibodies, both alone and in combination, against experimental infection with *V. cholerae* El Tor strains in the infant mouse cholera model

Antibody prepn and dilution	No. of survivors/total[a] (PE, %)	
	174 (El Tor)	T19479 (El Tor)
MSHA pilus antiserum		
1:5	3/4 (75)**	4/5 (79)**
1:12	5/7 (71)**	3/5 (57)§
1:15	5/15 (33)§	4/10 (36)§
1:20	1/10 (10)§	2/5 (36)§
LPS Inaba antiserum		
1:5	5/5 (100)**	NT[b]
1:10	4/5 (80)**	4/5 (79)**
1:20	4/10 (40)§	4/10 (36)§
1:30	0/5 (0)	2/5 (36)§
Preimmune sera (1:30)	0/10	1/15
MSHA pilus antiserum (1:15) plus LPS Inaba antiserum (1:20)	8/10 (80)**	8/10 (71)**

[a] **, $P < 0.01$; §, not significant ($P < 0.1$), both versus preimmune sera (Fisher's exact test).
[b] NT, not tested.

To determine whether anti-LPS antibodies (anti-LPS Inaba and anti-LPS Ogawa) had any protective effect against experimental El Tor cholera, Fab fragments of the rabbit antisera were also evaluated in the infant mouse model. Again, these antibodies were found to have a significant protective effect against both El Tor *V. cholerae* strains tested (Table 5). The protective effect was again Ig concentration dependent.

Because both anti-MSHA pilus and anti-LPS antibodies were able to protect baby mice against challenge with El Tor bacteria, a possible synergistic protective effect of the Fab fragments of these antibodies was evaluated. In this experiment, the concentrations of the specific antibody preparations used previously alone were again not sufficient to offer significant protection against challenge with the El Tor strains (for anti-MSHA pilus Fab fragments diluted 1:15, PEs = 33% [strain 174] and 36% [strain T19479]; for anti-LPS Fab fragments diluted 1:20, PEs = 36% [strain 174] and 40% [strain T19479]; Table 5). As shown in Table 5, the combination of both antibody preparations resulted in significantly stronger protection against challenge with both *V. cholerae* El Tor strains than did the Fab fragments of each antiserum alone (for strain 174, PE = 80%; for strain T19479, PE = 71%).

DISCUSSION

The ability of *V. cholerae* O1 strains to colonize the small intestine as well as to produce cholera toxin seems to be important for virulence. Two main colonization factors have been proposed to be involved in the colonization process: TCP for the classical and MSHA pili for the El Tor biotype. Since these adhesins are immunogenic (19, 32) and seem to be present on most classical (TCP) and El Tor (MSHA pili) vibrios, they are of potential interest for immunization against cholera. In this study, we have evaluated the protective capacity of various antifimbrial as well as anti-LPS antibody preparations, alone and in combination, against challenge with classical and El Tor vibrios. Our results support previous findings (29, 32, 33, 37) that antibodies against TCP may protect baby mice against experimental infection with classical biotype vibrios. Like Apter et al. (1), we also found that antibodies to the LPS component prevented infection with *V. cholerae* O1 strains and that this protective effect was specific and dose dependent. Moreover, our results indicate that antibodies against TCP fimbriae and against LPS cooperate, at least additively and possibly even synergistically, in protecting baby mice against challenge with classical vibrios. Neither preimmune serum nor nonspecific MAbs were protective, indicating that the specific antibodies were responsible for the protective effect of the immune serum or TCP MAbs. This was further supported by findings that the antibody preparations used in the study showed high specificity in recognizing fimbrial and *V. cholerae* LPS antigens, as determined in ELISA and immunoblot analyses.

We have also found that neither rabbit antisera nor mouse MAbs against TCP have a significant protective effect against challenge with *V. cholerae* O1 of the El Tor biotype. It was previously shown by Jonson et al. (18) that TCP fimbriae are poorly or not at all expressed on the surface of El Tor vibrios, although TcpA, the structural subunit of TCP, is produced by

V. cholerae O1 bacteria of both the classical and El Tor biotypes. Against this background, it is not surprising that antibodies against TCP did not protect baby mice against challenge with the El Tor *V. cholerae* O1 strains (Table 2). In this regard, our findings are similar to those described by Sharma et al. (30). They also observed that specific anti-TCP antibodies were able to protect mice against challenge with classical but not with El Tor vibrios. However, Sun et al. (32) claimed that mice infected with El Tor vibrios were also protected when antibodies to classical TCP were used at a high concentration. Our results show biotype-associated differences in the protective activity of specific antibodies against TCP.

It has also been reported that at least some strains of El Tor vibrios can express TCP-like bundles (20, 22) and that a mutation in the *tcpA* gene was associated with reduced colonizing ability of an El Tor strain in the infant mouse intestine (22). Since our TCP antibody preparations were raised against TCP from classical vibrios, we cannot exclude the possibility that the biotype-specific protection observed was due to inadequate reactivity of the immune reagents with protective El Tor TCP antigens. However, the polyclonal TCP antiserum used in the protection test reacted equally well with both classical and El Tor TcpA subunits, and one of the MAbs used (TCP 20:2) showed significant cross-reactivity with both classical and El Tor TcpA, as tested in immunoblot analyses, which makes this explanation less likely.

Similar but reverse biotype differences in protection against challenge with classical and El Tor vibrios were observed when antibodies against MSHA pili were used. The results of our study support previous findings that antibodies directed to this fimbrial antigen protect baby mice against experimental El Tor cholera but not against classical biotype cholera (25). The protection data are also consistent with previous results showing that MSHA fimbriae are usually expressed on the surface only of *V. cholerae* of the El Tor biotype even though the MSHA pilin protein is found intracellularly in bacteria of both biotypes (19). Although the polyclonal antiserum against MSHA pili also contained detectable amounts of antibodies to *V. cholerae* LPS, the protection obtained was most likely due to the specific antibodies against MSHA pili, since the anti-LPS antibody titer was <0.1% of the anti-MSHA pilus titer and thus was not sufficient for protective activity (see Table 1). Furthermore, the specific protective effect of MSHA pilus antibodies was further supported by our finding that MAb 17:10 substantially reduced mortality among mice infected with El Tor but not with classical vibrios. The specificity of the MAb ascites fluid was demonstrated in both ELISA and immunoblot analyses.

The results of our study demonstrate that in the experimental cholera system, antifimbrial and anti-LPS antibodies showed an additive or truly synergistic cooperation for protection against challenge with *V. cholerae* O1 strains. This synergistic effect seems to be due to actions at different mucosal levels. Anti-LPS antibodies may prevent colonization by decreasing motility, since LPS is also found on flagella (8, 31), and it seems that these antibodies mediate their protective effect primarily through inhibition of intestinal colonization, since Fab fragments of the specific Igs used in our test were strongly protective, although they were not able to agglutinate bacterial cells. Furthermore, antifimbrial antibodies may block the final adhesion of vibrios to enterocytes. Thus, despite the well-known cooperation between anti-LPS and antitoxin antibodies at different pathogenic levels (i.e., mucosal adhesion of bacteria and binding of cholera toxin), antibodies against non-LPS antigens (i.e., TCP and MSHA pili) are also capable of synergistic cooperation with anti-LPS Igs for protection.

The results of our study indicate that TCP and MSHA pili may play an important role in the pathogenesis of experimental cholera caused by *V. cholerae* O1 of the classical and of the El Tor biotype, respectively. Specific antibody preparations directed to TCP and MSHA pilus antigens were able to protect against challenge with classical and El Tor vibrios, respectively. Our results indicate that antibacterial immunity preventing colonization is complex and involves immune reactions with both LPS and fimbrial epitopes. We have found that the addition of anti-LPS antibodies to the antifimbrial Igs resulted in a protective effect against *V. cholerae* O1 bacteria which corresponded to or exceeded the sum of the PEs obtained with each antibody preparation alone. This cooperative protective activity might be utilized to generate new oral cholera vaccines including both TCP and MSHA fimbrial antigens.

ACKNOWLEDGMENTS

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 Programme for Vaccine Development.

REFERENCES

1. **Apter, F. M., P. Michetti, L. S. Winner III, J. A. Mack, J. J. Mekalanos, and M. R. Neutra.** 1993. Analysis of the roles of antilipopolysaccharide and anti-cholera toxin immunoglobulin A (IgA) antibodies in protection against *Vibrio cholerae* and cholera toxin by use of monoclonal IgA antibodies in vivo. Infect. Immun. **61**:5279–5285.
2. **Attridge, S. R., and D. Rowley.** 1983. Prophylactic significance of the non-lipopolysaccharide antigens of *Vibrio cholerae*. J. Infect. Dis. **148**:864–872.
3. **Baselski, V., R. Briggs, and C. Parker.** 1977. Intestinal fluid accumulation induced by oral challenge with *Vibrio cholerae* or cholera toxin in infant mice. Infect. Immun. **15**:704–712.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Clemens, J. D., D. A. Sack, J. R. Harris, F. van Loon, J. Chakraborty, F. Ahmed, M. R. Rao, M. R. Khan, M. D. Yunus, N. Huda, B. N. Stanton, B. A. Kay, S. Walter, R. Eeckels, A.-M. Svennerholm, and J. Holmgren.** 1990. Field trial of oral cholera vaccines in Bangladesh: results from three-year follow-up. Lancet **335**:270–273.
6. **De St. Groth, S. F., and D. J. Scheidegger.** 1980. Production of monoclonal antibodies: strategy and tactics. J. Immunol. Methods **35**:1–21.
7. **Ehara, M., M. Ishibashi, Y. Ichinose, M. Iwanaga, S. Shimotori, and T. Naito.** 1987. Purification and partial characterization of fimbriae of *Vibrio cholerae* O1. Vaccine **5**:283–288.
8. **Fuerst, J. A., and J. W. Perry.** 1988. Demonstration of lipopolysaccharide on sheathed flagella of *Vibrio cholerae* O:1 by protein A-gold immunoelectron microscopy. J. Bacteriol. **170**:1488–1494.
9. **Hall, R. H., P. A. Vial, J. B. Kaper, J. J. Mekalanos, and M. M. Levine.** 1988. Morphological studies on fimbriae expressed by *Vibrio cholerae* O1. Microb. Pathog. **4**:257–265.
10. **Hall, R. M., A. D. P. Silveira, and P. A. Vial.** 1990. Expression, purification and analysis of toxin co-regulated pili from classical *Vibrio cholerae* O1, abstr. B-85, p. 40. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990.
11. **Hanne, F., and R. A. Finkelstein.** 1982. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. Infect. Immun. **36**:209–214.
12. **Herrington, D. A., R. H. Hall, G. Losonsky, J. J. Mekalanos, R. K. Taylor, and M. M. Levine.** 1988. Toxin, toxin-coregulated pili, and the *tox*R regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med. **168**:1487–1492.
13. **Holmgren, J.** 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature (London) **292**:413–417.
14. **Holmgren, J., A.-M. Svennerholm, I. Lönnroth, M. Fall-Persson,**

M. Markman, and H. Lundbeck. 1977. Development and improved cholera vaccine based on subunit toxoid. Nature (London) **269**:602–604.
15. **Iwanaga, M., N. Nakasone, T. Yamashiro, and N. Higa.** 1993. Pili of *Vibrio cholerae* widely distributed in serogroup O1 strains. Microbiol. Immunol. **37**:23–28.
16. **Iwanaga, M., K. Yamamoto, N. Higa, Y. Ichinose, N. Nakasone, and M. Tanabe.** 1986. Culture conditions for stimulating cholera toxin production by *Vibrio cholerae* O1 El Tor. Microbiol. Immunol. **30**:1075–1083.
17. **Jacob, A., V. B. Sinha, M. K. Sahib, R. Srivastava, J. B. Kaper, and B. S. Srivastava.** 1993. Identification of a 33 kDa antigen associated with an adhesive and colonizing strain of *Vibrio cholerae* El Tor and its role in protection. Vaccine **11**:376–382.
18. **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.
19. **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.
20. **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.
21. **Jonson, G., J. Sanchez, and A.-M. Svennerholm.** 1989. Expression and different biotype-associated cell-bound haemagglutinins of *Vibrio cholerae* O1. J. Gen. Microbiol. **135**:111–120.
22. **Kaufman, M. R., and R. K. Taylor.** 1994. The toxin-coregulated pilus: biogenesis and function, p. 187–202. *In* I. K. Wachsmuth, P. A. Blake, and Ø. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. American Society for Microbiology, Washington, D.C.
23. **Manning, P. A.** 1991. Molecular analysis of potential adhesions of *Vibrio cholerae* O1, p. 139–145. *In* T. Wadström, P. H. Mäkelä, A.-M. Svennerholm, and H. Wolf-Watz (ed.), Molecular pathogenesis of gastrointestinal infections. Plenum Press, New York.
24. **Ørskov, F., I. Ørskov, B. Jann, E. Muller-Seitz, and O. Westphal.** 1967. Immunochemistry of *Escherichia coli* O antigens. Acta Pathol. Microbiol. Scand. Sect. B **71**:339–358.
25. **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.
26. **Pierce, N. F., W. C. Cray, Jr., and J. H. Saci.** 1982. Oral immunization of dogs with purified cholera toxin, crude cholera toxin, or B subunit: evidence for synergistic protection by antitoxin and antibacterial mechanisms. Infect. Immun. **37**:687–694.
27. **Porter, R. R.** 1959. The hydrolysis of rabbit immunoglobulin and antibodies with crystalline papain. Biochem. J. **73**:119–126.
28. **Sengupta, D. K., T. K. Sengupta, and A. C. Ghose.** 1992. Major outer membrane proteins of *Vibrio cholerae* and their role in induction of protective immunity through inhibition of intestinal colonization. Infect. Immun. **60**:4848–4855.
29. **Sharma, D. P., S. Attridge, J. Hackett, and D. Rowley.** 1987. Non-lipopolysaccharide protective antigens shared by classical and El Tor biotypes of *Vibrio cholerae*. J. Infect. Dis. **155**:716–723.
30. **Sharma, D. P., C. Thomas, R. H. Hall, M. M. Levine, and S. R. Attridge.** 1989. Significance of toxin-coregulated pili as protective antigens of *Vibrio cholerae* in the infant mouse model. Vaccine **7**:451–456.
31. **Sinha, V. B., A. Jacob, R. Srivastava, J. B. Kaper, and B. S. Srivastava.** 1993. Identification of the flagellar antigens of *Vibrio cholerae* El Tor and their role in protection. Vaccine **11**:372–375.
32. **Sun, D., J. J. Mekalanos, and R. K. Taylor.** 1990. Antibodies directed against the toxin-coregulated pilus isolated from *Vibrio cholerae* provide protection in the infant mouse experimental cholera model. J. Infect. Dis. **161**:1231–1236.
33. **Sun, D., D. M. Tillman, T. N. Marion, and R. K. Taylor.** 1990. Production and characterization of monoclonal antibodies to the toxin coregulated pilus (TCP) of *Vibrio cholerae* that protect against experimental cholera in infant mice. Serodiagn. Immunother. Infect. Dis. **4**:73–81.
34. **Svennerholm, A.-M.** 1975. Experimental studies on cholera immunization. IV. The antibody response to formalinized *V. cholerae* and purified endotoxin with special reference to protective capacity. Int. Arch. Allergy **49**:434–452.
35. **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.
36. **Svennerholm, A.-M., G. Jonson, and Y. Chang.** 1991. A method for studies of an El Tor-associated antigen of *Vibrio cholerae* O1. FEMS Microbiol. Lett. **79**:179–186.
37. **Taylor, R. K., V. L. Miller, D. B. Furlong, and J. J. Mekalanos.** 1987. Use of *pho*A gene fusion to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA **84**:2833–2837.