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NOTES

Antibody against the Capsule of *Vibrio cholerae* O139 Protects against Experimental Challenge

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Antiserum to the capsular polysaccharide of an opaque variant of *Vibrio cholerae* O139 strain MDO-12 recognizes capsular antigen in three different colonial variants of the strain, although the amount of recognition varies with the extent of opacity. The anti-capsular-polysaccharide serum, at subagglutinating doses, protected suckling mice against challenge with both the most opaque variant and the most translucent variant. Further studies indicated that the protection was associated with inhibition of intestinal colonization by the vibrios. These results thus highlight the potential importance of the capsule in immunoprophylaxis against cholera caused by *V. cholerae* O139.

Cholera is a severe and sometimes lethal secretory diarrheal disease caused by the bacterium *Vibrio cholerae*. Until 1992, only *V. cholerae* of the O1 serovar was considered to cause epidemic cholera. *V. cholerae* strains of non-O1 serovars have been associated, infrequently, with sporadic cases and limited outbreaks of diarrheal disease in humans (4). But in October 1992, an unprecedented change in cholera occurred on the Indian subcontinent with the emergence of massive epidemics of the disease caused by non-O1 *V. cholerae* (1, 2, 21): the newly described *V. cholerae* O139 synonym Bengal (24). The rapid spread of *V. cholerae* O139 among people of all ages in regions of high-level endemicity (where adults are relatively resistant) indicated that preexisting immunity to O1 cholera is not protective against O139 (6, 19).

Earlier studies have indicated that immunity to cholera is predominantly antibacterial rather than antitoxic in nature (17). The major differences between *V. cholerae* O1 and O139 reside in their cell surface components. *V. cholerae* O139 differs from O1 by its inability to produce the specific O1 lipopolysaccharide somatic antigen (24) and by its ability to express capsular polysaccharide (7, 16, 26, 27), which is not seen in O1 strains. The capsular polysaccharide, by virtue of its superficial location, may be expected to play a key role in the host-bacterium interaction and possibly to contribute to the virulence of O139 strains. Preliminary information regarding the structure and chemical composition of the capsular polysaccharide has been reported (20, 27). Volunteers who had recovered from induced cholera due to *V. cholerae* O139 (18) or who had received certain attenuated *V. cholerae* O139 mutant vaccine candidates (8) were protected against rechallenge or challenge with wild-type O139. Antibodies to cholera toxin, lipopolysaccharide, and capsular polysaccharide were detected in the sera of those volunteers. In one study (8) sera from vaccinees were reported to be vibriocidal, whereas in the other (18) they were not. The present study indicates that antibody to capsular polysaccharide is protective against experimental cholera caused by O139 *V. cholerae.*

The *V. cholerae* strain used in this study, MDO-12, was isolated from a cholera patient in Madurai, India, and it was provided by G. Balakrish Nair. The culture of this strain, when received and examined by us, yielded three different colony types, A, B, and C, in order of increasing opacity, which were selected for this study. The opacities of these three variants were quantified, in terms of opacity units (OUs) on a scale of 10, by using a stereoscope and transmitted oblique illumination (12), by comparison with a set of reference cultures with opacities of 2, 4, 6, and 8 OU. For this purpose, the reference cultures were dilution plated as 10-μl droplets to obtain isolated colonies near the center of the petri dish (11). Unknown cultures, similarly diluted and plated around the periphery, were classified by comparison with the reference cultures. The most translucent variant, MDO-12-A, has an opacity of 2 OU; MDO-12-B has an opacity of 4 OU; and the most opaque variant, MDO-12-C, has an opacity of 10 OU. All the strains were maintained at −70°C in tryptic soy broth containing 20% (vol/vol) glycerol. They were grown at 37°C on a modified "2/1" agar medium (11) containing Difco peptone (1%), Difco beef extract (0.5%), NaCl (0.5%), glycerol (0.5%), and agar (2%), omitting the glucose in the original formulation.

Capsular polysaccharide, used to raise antibody, was prepared from the most opaque variant, MDO-12-C, by following the method of Johnson et al. (15). Briefly, harvested bacteria were suspended in 0.5× phosphate-buffered saline and shaken at 200 rpm on a rotary shaker for 2 h at room temperature. The bacterial suspensions were then centrifuged (16,000 × *g*, 20 min, 4°C), and the supernatants were dialyzed with multiple changes of distilled water. The samples were ultracentrifuged (154,000 × *g*, 2 h, 20°C), and the supernatant was retained and digested with RNase and DNase and finally deproteinized with pronase E. The capsular polysaccharide was quantified by the phenol-sulfuric acid method of Dubois et al. (9).

Antisera to the purified capsular antigen were prepared by intramuscular immunization of two rabbits at 2-week intervals. The first injection was performed with about 100 μg of purified capsular antigen emulsified with Freund's complete adjuvant, and subsequent immunizations were done with the same amount of antigen emulsified with Freund's incomplete adjuvant. Four to five such immunizations were done, and sera collected 10 days after the last immunization were pooled and decomplemented (56°C, 30 min) prior to use in protection

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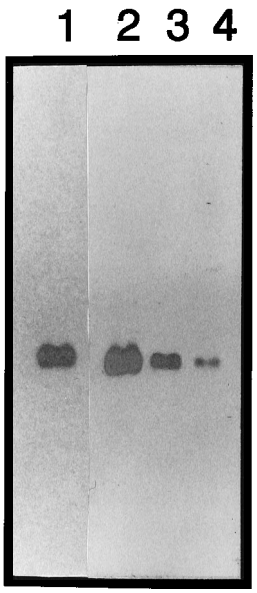

FIG. 1. Western blot analysis with serum against purified capsular antigen (2 μg) of opaque variant MDO-12-C (lane 1) and lysates of 10^7 bacteria (35 μg of protein) of spontaneous phase variants MDO-12-C, MDO-12-B, and MDO-12-A (lanes 2 to 4, respectively).

studies. The purity of the preparation and the specificity of the antiserum were demonstrated by Western immunoblotting, modified from the method of Burnette (5). Only a single immunoreactive band was demonstrated (Fig. 1, lane 1) in purified polysaccharide or in whole bacterial lysates (Fig. 1, lanes 2 to 4). Parallel gels of the lysates, stained with Coomassie blue, revealed the usual, but overloaded, pattern of numerous protein bands throughout the gel (result not shown). The anti-capsular-polysaccharide antibody did not stain lipopolysaccharide purified by the technique of Westphal and Jann (28), which was demonstrated by silver staining (25). The antibody also did not stain, by immunofluorescence or by Western blotting, an acapsular mutant derived from MDO-12-C which still agglutinated with O139 antiserum (22).

Electron microscopic studies in our laboratory of the colonial variants, using polycationic ferritin (15), demonstrated that the most opaque variant, MDO-12-C, was heavily encapsulated, while the most translucent one, MDO-12-A, possessed only a small amount of capsular material (11). The electron microscopic observations (11) were extended in the present study by performing Western immunoblotting (5) with anti-capsular-polysaccharide serum by using bacterial lysate as the antigen (Fig. 1). The most opaque variant, MDO-12-C, exhibited a large amount of immunoreactive capsular antigen. MDO-12-B also showed a strong reactive band with anti-capsular-polysaccharide serum, while the most translucent variant, MDO-12-A, had very weak reactivity with anti-capsular-polysaccharide antiserum. In contrast to the report of Waldor et al. (26), our observations showed no immunologic relatedness between the capsular polysaccharide and the lipopolysaccharide somatic antigen, as the anti-capsular-polysaccharide serum failed to react with lipopolysaccharide or other components of the vibrio lysates.

Prior to use in protection studies, the anti-capsular-polysaccharide serum was assayed for bacterial agglutination. Bacterial suspensions (2×10^8 CFU/0.5 ml) were added to equal volumes of serially twofold-diluted antiserum in glass tubes (1.2 by 10 cm), and the mixtures were incubated for 2 h at 37°C and then overnight at 4°C. The agglutination titer was expressed as the highest dilution of the antiserum showing agglutination visible to the naked eye.

TABLE 1. Protective activity of anti-capsular-polysaccharide serum against challenge with *V. cholerae* O139 strains in the suckling-mouse model

Challenge strain[a]	Preincubation medium	Antiserum dilution	Fluid accumulation ratio[b]
MDO-12-C	0.1% Peptone saline		0.103 (0.008)
	Preimmune serum[c]	1:100	0.096 (0.007)
	Anti-capsular-polysaccharide serum[d]	1:800	0.067 (0.002)
		1:1,600	0.083 (0.004)
MDO-12-A	0.1% Peptone saline		0.098 (0.003)
	Preimmune serum[c]	1:100	0.093 (0.005)
	Anti-capsular-polysaccharide serum[d]	1:100	0.073 (0.003)
		1:200	0.096 (0.003)

[a] Each mouse was challenged with 10^8 CFU of bacteria suspended in 0.1 ml of 0.1% peptone saline with or without anti-capsular-polysaccharide serum.
[b] Calculated as gut weight/(body weight − gut weight) 16 h after oral challenge. Results are the means for six mice, with standard errors in parentheses.
[c] Preimmune serum did not show any agglutination of *V. cholerae.*
[d] Two- and fourfold subagglutinating dilutions were used.

The protective activity of anti-capsular-polysaccharide serum against live-vibrio challenge was determined by passive protection experiments with the suckling-mouse model (23). For this, 3- to 4-day-old CFW mice were initially starved for 6 h prior to use to ensure that their stomachs did not contain any milk. Each group of six animals was inoculated orally with 0.1 ml containing about 10^8 CFU of vibrios which had been preincubated at 37°C for 30 min with subagglutinating (two- and fourfold) dilutions of anti-capsular-polysaccharide serum. Groups of mice which received vibrio suspension alone (i.e., without any antiserum) or with preimmune serum served as negative controls for protection. Sixteen hours after challenge, the mice were sacrificed and their body weights and gut weights were measured. Results were then expressed as the fluid accumulation ratio, calculated as gut weight/(body weight − gut weight) (3).

The results (Table 1) showed that the anti-capsular-polysaccharide serum, at subagglutinating doses, was protective, as indicated by the lower fluid accumulation ratios following challenge. The titer of antibody protective against the more heavily encapsulated variant, MDO-12-C, was higher, but moderate protection was also induced by the anti-capsular-polysaccharide serum, at one-half but not one-fourth of an agglutinating dose, against challenge with the translucent variant MDO-12-A. Preimmune serum was not protective.

The anti-capsular-polysaccharide serum was further used to test the inhibition of intestinal colonization of *V. cholerae* in the suckling-mouse model (23). For this, each group of animals was challenged orally with 0.1 ml of vibrio suspension containing approximately 10^5 CFU of variant MDO-12-C, which was preincubated at 37°C for 30 min with subagglutinating dilutions of anti-capsular-polysaccharide serum. Sixteen hours after oral challenge, the animals were sacrificed and their intestines were removed, washed thoroughly to remove free bacteria, and homogenized separately. Viable counts for each of the homogenates were determined by dilution plating. Groups of mice which received vibrio suspension alone (i.e., without any antiserum) or with preimmune serum served as negative controls.

The colonization experiment showed that the viable counts of adherent MDO-12-C had increased nearly 300-fold by 16 h following vibrio challenge in the control mice (Table 2). The anti-capsular-polysaccharide serum at two- and fourfold sub-

TABLE 2. Inhibition of intestinal colonization of opaque variant of *V. cholerae* O139 strain MDO-12-C by anti-capsular-polysaccharide serum in suckling mice[a]

Preincubation medium	Antiserum dilution	CFU/intestine[b]
0.1% Peptone saline		3.2×10^7 (0.023×10^7)
Preimmune serum[c]	1:100	2.8×10^7 (0.21×10^7)
Anti-capsular-polysaccharide serum[d]	1:800	3.9×10^4 (0.26×10^4)
	1:1,600	8.7×10^5 (0.67×10^5)

[a] Each mouse was challenged with 1.3×10^5 CFU of bacteria suspended in 0.1 ml of 0.1% peptone saline with or without anti-capsular-polysaccharide serum.
[b] Determined after 16 h of vibrio challenge. Results are mean counts of the bacteria remaining on the intestinal tissue after the wash per group of three mice, with standard errors in parentheses.
[c] Preimmune serum did not show any agglutination of *V. cholerae.*
[d] Two- and fourfold subagglutinating dilutions were used.

agglutinating dilutions inhibited intestinal colonization 820- to 37-fold, respectively, compared with the negative control. Preimmune serum had no such inhibitory activity.

The anti-capsular-polysaccharide serum was also tested for vibriocidal activity (10) against strain MDO-12-C in parallel with a diagnostic antiserum raised against *V. cholerae* O139 strain MO-45 (24). Whereas the anti-capsular-polysaccharide serum had no vibriocidal activity, the anti-O139 serum had a very low titer, i.e., 10^{-1}, in this extremely sensitive assay (10).

Results presented in this study thus establish the potential importance of antibody to capsular antigen in protection against cholera. This study also demonstrates that such protection by anti-capsular-polysaccharide serum is associated with inhibition of intestinal adherence of *V. cholerae*. The fact that subagglutinating dilutions of the antiserum were used indicates that the observed inhibition of colonization and subsequent protection were not the consequence of bacterial clumping, and it should be noted that the serum lacked complement-dependent vibriocidal activity.

Our results support the notion that the capsular polysaccharide of *V. cholerae* O139 is likely to play a significant role in immunity. The capsular polysaccharide should be considered in strategies for the development of an effective vaccine against *V. cholerae* O139. A parenterally administered capsular polysaccharide-cholera toxin (or cholera toxin analog [14]) conjugate, along the lines suggested by Gupta et al. (13), would be worthy of further study.

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