Vibrio cholerae O139 Conjugate Vaccines: Synthesis and Immunogenicity of *V. cholerae* O139 Capsular Polysaccharide Conjugates with Recombinant Diphtheria Toxin Mutant in Mice

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Epidemiologic and experimental data provide evidence that a critical level of serum immunoglobulin G (IgG) antibodies to the surface polysaccharide of *Vibrio cholerae* O1 (lipopolysaccharide) and of *Vibrio cholerae* O139 (capsular polysaccharide [CPS]) is associated with immunity to the homologous pathogen. The immunogenicity of polysaccharides, especially in infants, may be enhanced by their covalent attachment to proteins (conjugates). Two synthetic schemes, involving 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) as activating agents, were adapted to prepare four conjugates of *V. cholerae* O139 CPS with the recombinant diphtheria toxin mutant, CRMH21G. Adipic acid dihydrazide was used as a linker. When injected subcutaneously into young outbred mice by a clinically relevant dose and schedule, these conjugates elicited serum CPS antibodies of the IgG and IgM classes with vibriocidal activity to strains of capsulated *V. cholerae* O139. Treatment of these sera with 2-mercaptoethanol (2-ME) reduced, but did not eliminate, their vibriocidal activity. These results indicate that the conjugates elicited IgG with vibriocidal activity. Conjugates also elicited high levels of serum diphtheria toxin IgG. Convalescent sera from 20 cholera patients infected with *V. cholerae* O139 had vibriocidal titers ranging from 100 to 3,200; absorption with the CPS reduced the vibriocidal titer of all sera to ≤ 50 . Treatment with 2-ME reduced the titers of 17 of 20 patients to ≤ 50 . These data show that, like infection with *V. cholerae* O1, infection with *V. cholerae* O139 induces vibriocidal antibodies specific to the surface polysaccharide of this bacterium (CPS) that are mostly of IgM class. Based on these data, clinical trials with the *V. cholerae* O139 CPS conjugates with recombinant diphtheria toxin are planned.

It has been proposed that a critical level of serum immunoglobulin G (IgG) to the surface polysaccharides of *Vibrio cholerae* O1 and *V. cholerae* O139 confers serotype-specific immunity to cholera (3, 7, 17, 24, 25, 28, 29–32, 38, 39, 43, 44). The surface polysaccharide of *V. cholerae* O1 is a lipopolysaccharide (LPS). *V. cholerae* O139, in contrast, has a capsular polysaccharide (CPS) composed of a hexasaccharide repeating unit containing a trisaccharide backbone and two branches (3, 4, 8, 10, 12, 16, 17, 19, 26, 28, 30, 31, 35, 38, 42, 43, 45). The repeating unit contains two negatively charged groups: a carboxyl of galactouronic acid and a phosphate cyclic diester. In our preliminary studies we found that CPS did not elicit serum antibodies after three injections in mice (Z. Kossaczka and S. C. Szu, submitted for publication). To improve its immunogenicity, CPS was covalently bound by different synthetic schemes to chicken serum albumin, as a model protein. The resultant conjugates induced serum anti-CPS IgG in mice with vibriocidal activity (Kossaczka and Szu, submitted).

We chose the two most successful schemes to prepare *V. cholerae* O139 CPS conjugates with the recombinant diphtheria toxin (rDT) mutant CRMH21G. CRMH21G was prepared

by replacing histidine 21 with glycine in the A chain of DT (15). This mutant protein has a 10^{-4} lower toxicity than DT and is suitable for clinical use.

MATERIALS AND METHODS

Materials. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), adipic acid dihydrazide (ADH), 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), and agarose were from Sigma Chemical Co., St. Louis, Mo.; Sepharose CL-4B and Sephadex G-25 were from Pharmacia AB, Uppsala, Sweden; bovine serum albumin (BSA) standard solution, Coomassie blue protein assay reagent, and triethylamine (TEA) were from Pierce, Rockford, Ill.; nickel-nitrilotriacetic acid (Ni-NTA) chelating agarose was from Qiagen, Inc., Chatsworth, Calif.; acetonitrile was from T. J. Baker, Inc., Phillipsburg, N.J.; DT was from List Biological Laboratories, Inc., Campbell, Calif.; equine anti-DT serum from lot 152-5456 R was a gift from the Center for Biologics Evaluation and Research, U.S. Food and Drug Administration; rabbit (3 to 4 weeks old) complement was from Pel-Freez, Brown Deer, Wis.; dialysis membranes (molecular weight cut-off, 6,000 to 8,000) were from Spectra-Por, Laguna Hills, Calif.; ultrafiltration membrane YM100 and Centrprep 30 were from Amicon, Inc., Beverly, Mass.; *Limulus* amoebocyte lysate pyrogen (U.S. license no. 709) was from BioWhittaker, Inc., Walkersville, Md.; tryptic soy broth (TSB) was from Difco Inc., Detroit, Mich. (TSB containing 1% agarose was denoted as TSA). Deionized or pyrogen-free water (PFW) and pyrogen-free saline (PFS) were used in all experiments.

Bacteria. *V. cholerae* O139 MDO-12C (8), a heavily capsulated and opaque variant selected from the isolate MDO-12 (Madurai, India), was used for the preparation of CPS and murine hyperimmune serum. *V. cholerae* O139 SPH1168, a clinical isolate from a Thai patient (Suanphung Hospital, Suanphung, Thailand) (13), was used as the target strain in the vibriocidal assay. Both isolates were stored in 20% glycerol at -70°C .

Purification of *V. cholerae* O139 CPS. *V. cholerae* O139 MDO-12C was propagated from a single colony on TSA to 400 ml and then to 4 liters of TSB for 5 h

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at 37°C with shaking at 200 rpm. The 4-liter inoculum ($A_{560} \sim 3.0$) was transferred to a 300-liter fermentor containing 150 liters of TSB, 0.1% dextrose, and 0.05 M $MgSO_4$. Fermentation was conducted in 30% dissolved oxygen at 35°C and pH 7.0 (maintained with NH_4OH). After 16 h, formalin was added to a final concentration of 2%, and the mixture was stirred slowly for 6 h at room temperature. The suspension was centrifuged, and the supernatant concentrated to 1.2 liters by ultrafiltration and stored at $-20^\circ C$.

A 500-ml aliquot of the concentrated supernatant was mixed with 3 volumes of 95% ethanol and stored overnight at 4°C. The supernatant was decanted, and the slurry was spun down at $10,500 \times g$ at 10°C for 30 min. The pellet (20 g [wet weight]) was washed with 80% ethanol, dissolved in 800 ml of 10% saturated sodium acetate (pH 7.5), and extracted with cold phenol three times (9). The final water phase was dialyzed against H_2O for 3 days at 4 to 8°C and freeze-dried. The precipitate was dissolved in 150 ml of 0.1 M $CaCl_2$ and ultracentrifuged at $145,000 \times g$ at 10°C for 5 h. The supernatant was recentrifuged as described above, dialyzed against H_2O , freeze-dried (yield, 1.6 g), and stored at $-20^\circ C$.

This material (unfractionated CPS) was dissolved in PFW (100 mg/50 ml) and passed through an Amicon membrane YM100. The retentate was passed through a 2.5-by-90-cm column of Sepharose CL-4B in PFS. The retentate was eluted from the column as one peak at K_d 0.4; fractions were pooled, dialyzed against PFW, and freeze-dried. This material was denoted CPS and used to prepare conjugates with rDT.

^{13}C NMR spectroscopy. The ^{13}C NMR spectrum of the CPS (50 mg/ml of D_2O) was measured using a Varian XL3000 spectrometer by averaging 50,000 scans with a 10-s decay between acquisition and a $10\text{-}\mu s$ 90° pulse. Prior to Fourier transformation, a 5-Hz line broadening was applied and zero filled to 32,000 datum points.

Murine hyperimmune *V. cholerae* O139 serum. *V. cholerae* O139 culture was prepared by transferring a single colony from TSA to 50 ml of LB and incubating it at 37°C at 200 rpm for 5 h ($A_{560} \sim 1.0$). The culture was inactivated with 1% formalin. Thirty 6-week-old female Swiss mice (National Institutes of Health) were injected as follows: (i) three subcutaneous injections of 100 μl 1 day apart; (ii) after 9 days, 3 intraperitoneal injections of 150 μl 1 day apart; and (iii) 9 days later, three intravenous injections of 200 μl 1 day apart. Mice were exsanguinated 7 days after the last injection. All sera showed a precipitin line by double immunodiffusion with CPS; a pool was denoted as murine hyperimmune *V. cholerae* O139 serum.

Purification of rDT mutant CRMH21G. The rDT mutant was constructed by site-directed mutagenesis on the A chain, replacing histidine at position 21 with glycine, and expressed in *Escherichia coli* BL21(λ DE3) (15). To facilitate purification by use of Ni-NTA, a six-histidine tag was attached to the protein carboxyl terminal. Fermentation of this recombinant strain was performed as described previously (5). The cell paste was suspended in 0.5 M NaCl–0.02 M Tris–0.005 M imidazole (pH 8.0), and the cytoplasm was released by a French press. The supernatant was passed through a 2.5-by-10-cm Ni-NTA column and washed with 0.02 M Tris buffer containing 0.03 M imidazole (pH 8.0). rDT was eluted with 0.02 M Tris buffer containing 0.25 M imidazole (pH 8.0) at 3 to 8°C. The eluate was dialyzed exhaustively against 0.02 M Tris (pH 8.0) at 3 to 8°C (yield, 150 mg of rDT/liter of supernatant). Prior to derivatization, rDT was dialyzed at 3 to 8°C against phosphate-buffered saline with multiple changes of outer fluid, followed by dialysis against 0.2 M NaCl (pH 7.2 to 7.7; adjusted with 1 M NaOH). The protein solution was concentrated to ~ 10 mg/ml in an Amicon Centriprep 30.

rDT had the same R_f by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in a 10% polyacrylamide gel and the same circular dichroism spectrum as DT (not shown). A line of identity was formed between rDT and DT when they were reacted against equine anti-DT serum by double immunodiffusion (not shown).

Adipic acid hydrazide (AH) derivatives. CPS and rDT were treated with ADH in the presence of CDAP (20, 21, 23) and EDC (22, 37), respectively, as activating agents.

AH derivative of CPS (CPS_{AH}). CDAP activation of CPS was performed as described earlier (21) using a CDAP/CPS ratio of 1:5 (wt/wt). A total of 40 mg of CPS was dissolved in 1.5 ml of H_2O (pH 5.0). Then, 80 μl of CDAP in acetonitrile (100 mg/ml) was added, followed in 30 s by adding 80 μl of 0.2 M TEA. After 2 min, the pH was adjusted to 7.8 with 0.1 M HCl. Then, 1.5 ml of 0.8 M ADH in 0.5 M $NaHCO_3$ was added, and the pH was maintained between 8.0 and 8.4 with 0.1 M HCl for 2 h at room temperature. The reaction mixture was dialyzed overnight against 6 liters of water with two changes and then passed through a 2.5-by-40-cm column of Sephadex G-25 in PFW. The void volume fractions, denoted CPS_{AH}, were freeze-dried and assayed for AH.

AH derivative of rDT (rDT_{AH}). The reaction mixture contained 10 mg of protein per ml, 0.2 M ADH, and 0.011 M EDC. The reaction was carried out for 1 h at room temperature, with the pH maintained at 6.2 to 6.4 with 0.1 M HCl. The reaction mixture was dialyzed against 0.2 M NaCl (pH 7.0; adjusted with 1 M NaOH) and passed through a 1.5-by-25-cm column of Sephadex G-25 in the same solution. Void volume fractions, denoted rDT_{AH}, were pooled, concentrated (Amicon Centriprep 30), and assayed for protein and AH.

Conjugates. Two schemes were used to prepare conjugates: EDC-mediated conjugation of the CPS_{AH} with rDT and CDAP-mediated conjugation of the CPS with rDT_{AH}.

(i) **EDC-mediated conjugation of CPS_{AH} with rDT.** Each reaction mixture contained 8 mg of CPS_{AH} and rDT per ml and EDC at 0.05 M (for I:CPS_{AH}-rDT) or 0.02 M (for II:CPS_{AH}-rDT).

CPS_{AH} was dissolved in 0.2 M NaCl, and the pH was adjusted to 6.2 with 0.1 M NaOH. rDT was added, and the volume was adjusted with 0.2 M NaCl. After stirring the mixture for 1 min, the EDC was added. The reaction was carried out for 3 h at room temperature, and the pH was maintained at 6.2 to 6.4 with 0.1 M HCl. The mixture was dialyzed overnight at 3 to 8°C against 0.2 M NaCl–0.005 M sodium phosphate (pH 7.5) and passed through a 1.5-by-90-cm column of Sepharose CL-4B in the same buffer. Fractions were assayed for polysaccharide and protein. The void volume fractions were pooled and denoted I:CPS_{AH}-rDT and II:CPS_{AH}-rDT.

(ii) **CDAP-mediated conjugation of CPS with rDT_{AH}.** Each reaction mixture contained 8 mg of CPS and rDT_{AH} per ml: the CDAP/CPS ratio was 4:5 (for I:CPS-rDT_{AH}) or 1:5 (for II:CPS-rDT_{AH}).

CDAP (100 mg/ml of acetonitrile) was added to CPS in 0.2 M NaCl (pH 5.2) and mixed for 30 s. A volume of 0.2 M TEA equal to that of CDAP was added. After 2 min, the pH dropped from 8.5 to 7.2 and rDT_{AH} was added. The pH was raised from 7.2 to 8.3 with 0.1 M NaOH. The reaction was carried out for 2 h at room temperature, during which the pH was stable. The mixture was dialyzed overnight against 0.2 M NaCl–0.005 M sodium phosphate buffer (pH 7.5) and passed through a 1.5-by-90-cm column of Sepharose CL-4B in the same buffer. Fractions were assayed for polysaccharide and protein, and void volume fractions were pooled and denoted I:CPS-rDT_{AH} and II:CPS-rDT_{AH}.

Chemical assays. Polysaccharide was assayed by measuring 3,6-dideoxyhexose (colitose) with the CPS as the standard (18). Protein was measured by Coomassie blue assay with BSA as the standard (2). The hydrazide content of CPS_{AH} and rDT_{AH} was measured by the TNBS method using ADH as the standard (14). The degree of derivatization was expressed as a percentage of AH and the mole/mole ratio of AH to polysaccharide or to protein.

Limulus amoebocyte lysate test. CPS was assayed for endotoxin by limulus amoebocyte lysate test. The FDA Reference Standard Endotoxin (lot EC-5) was used as a reference for the assay. The test conforms with the Food and Drug Administration guideline (41).

Immunodiffusion. Double immunodiffusion of the conjugates was performed in 1% agarose gel in 0.15 M NaCl with murine hyperimmune cholera O139 serum and equine DT antiserum.

Immunization of mice. Six-week-old female Swiss albino mice (10 per group) were injected subcutaneously three times at 2-week intervals with 100 μl of immunogen containing 2.5 μg of the CPS alone or as the conjugate. A control group received one injection of 100 μl of saline. Mice were exsanguinated 7 days after each injection, and sera were stored at $-20^\circ C$.

Enzyme-linked immunosorbent assay (ELISA). Flat-bottom 96-well microtiter plates (Nunc-Immuno) were coated with CPS (20 μg /ml of PBS) and kept overnight at room temperature. After a washing with 0.15 M NaCl, 0.1% Brij, and 3 mM sodium azide, plates were blocked with 1% BSA in PBS for 2 h at room temperature. The plates were washed and twofold serial dilutions of sera

FIG. 1. Sepharose CL-4B gel filtration profile of the unfractionated *V. cholerae* O139 CPS. The refractive index response is indicated by a broken line; the colitose-containing fractions are indicated by a solid line. A point of separation between the retentate and the filtrate by diafiltration (Amicon YM100) of the unfractionated CPS (arrow) is also noted. Distribution coefficients (K_d) are depicted at the top of each peak.

FIG. 2. ^{13}C NMR spectrum of *V. cholerae* O139 CPS. The ^{13}C NMR spectrum of the CPS (50 mg/ml of D_2O) was measured using a Varian XL3000 spectrometer by averaging 50,000 scans with a 10-s decay between acquisition and a 10- μs 90° pulse. Prior to Fourier transformation, a 5-Hz line broadening was applied and zero filled to 32,000 datum points.

in 1% BSA–0.1% Brij–PBS were added. Reference serum was assayed in triplicates and samples in duplicates. Plates were incubated overnight at room temperature and washed, and the alkaline phosphatase-labeled goat antibody specific to mouse IgG or IgM was added. After 4 h at room temperature, the plates were washed, and the 4-nitrophenylphosphate substrate (1 mg/ml in 1 M Tris-HCl–3 mM MgCl_2 [pH 9.8]) was added. A_{405} was measured by using an MRX Dynatech reader.

Anti-CPS IgG was measured in all murine sera; anti-CPS IgM was measured only in 11 representative sera from mice injected three times with II:CPS_{AH}-rDT or I:CPS-rDT_{AH}. Murine hyperimmune *V. cholerae* O139 serum was used as the reference for both anti-CPS IgG and IgM. This serum was arbitrarily assigned a value of 1,000 ELISA units (EU)/ml for IgG and 100 EU/ml for IgM upon the observation that a 1/20,000 dilution of anti-IgG and a 1/100 dilution of anti-IgM gave approximately the same A_{405} .

An analogous ELISA procedure was used to measure anti-DT IgG: plates were coated with DT (5 $\mu\text{g}/\text{ml}$), and a mouse serum with a high titer of anti-DT IgG was arbitrarily assigned a value of 1,000 EU served as the reference.

ELISA results were computed with an ELISA data processing program provided by the Biostatistics and Information Management Branch, Centers for Disease Control, based upon four parameters of logistic-log function using the Taylor Series Linearization Algorithm (34). Anti-CPS IgG and anti-DT IgG levels are expressed as geometric means.

Statistics. Comparisons of the geometric means were performed with the two-sided Student *t* test or Wilcoxon analysis.

Vibriocidal assay. Eleven representative sera from mice injected three times with II:CPS_{AH}-rDT or I:CPS-rDT_{AH}, and twenty convalescent sera from cholera patients infected with *V. cholerae* O139 (Samutskakorn Hospital, Samutskakorn, Thailand [13]) were assayed for vibriocidal activity before and after treatment with 0.1 M 2-mercaptoethanol (2-ME) for 30 min at 37°C (10, 27). The patient sera were also tested for vibriocidal activity after absorption with CPS in a vibriocidal antibody inhibition (VAI) assay (6).

Bacteria were prepared by transferring a single colony from TSA into 10 ml of TSB and incubating them for 2 to 3 h at 37°C with shaking at 180 rpm. Then, 100 μl of this inoculum was transferred to 10 ml of TSB and incubated with shaking (180 rpm) at 37°C until the culture reached an A_{560} of 0.2 to 0.24 (3.0×10^7 to 4.0×10^7 cells/ml). The bacterial suspension was diluted 10⁵-fold in Dulbecco buffer.

VAI assay was performed in sterile nonpyrogenic 24-well cell culture plates (Costar, Corning, N.Y.) by mixing equal volumes of serum, bacteria, and complement. The tested serum was twofold serially diluted in Dulbecco buffer (for VAI assay in 100 mg of CPS/ml of Dulbecco buffer), so that each well contained 100 μl . Next, 100- μl aliquots of the bacteria and of complement were added into each well. Plates were incubated for 1 h at 37°C with shaking. Two 100- μl aliquots from each well were transferred into empty wells, and 1 ml of TSA (46 to 48°C) was added to all three wells. Plates were incubated overnight at 37°C, and the colonies were counted. The vibriocidal titer was defined as the reciprocal of the highest serum dilution showing $\geq 60\%$ reduction in the number of colonies compared to the control (complement only) (25).

RESULTS

***V. cholerae* O139 CPS.** The *V. cholerae* O139 CPS isolated from culture supernatant (see Materials and Methods) showed three peaks at K_d s of 0.4, 0.71, and 0.91 on Sepharose CL-4B with yields of 70, 29, and 1%, respectively (Fig. 1). Colitose, a component of the CPS repeating unit, was detected only in the peak at K_d 0.4. Fast separation of this peak material from the lower-molecular-weight materials (K_d s 0.71 and 0.91) was ac-

TABLE 1. ADH derivatives (AH) of *V. cholerae* O139 CPS and of rDT

Derivative	Activating agent	AH content	
		%	mol/mol ^a
CPS _{AH}	CDAP	3.44	0.21
rDT _{AH}	EDC	1.90	7.12

^a CPS repeating unit, M_r 1,053; rDT, M_r ~67,000.

FIG. 3. Sepharose CL-4B gel filtration profiles of *V. cholerae* O139 CPS conjugates with rDT. A representative chromatograph of the CPS_{AH}-rDT conjugates prepared by EDC-mediated synthesis (A) or of CPS-rDT_{AH} conjugates prepared by CDAP-mediated synthesis (B) is shown. Symbols: ■, polysaccharide; ○, protein; ▼, K_d of CPS; ▽, K_d of rDT.

complicated by diafiltration of the unfractionated CPS through an Amicon ultrafiltration membrane (YM100). To confirm its purity, the retentate was passed through Sepharose CL-4B and only showed a peak of K_d 0.4. The ^{13}C NMR spectrum of the retentate (equivalent to the peak-material of K_d 0.4) (Fig. 2) was identical to a published ^{13}C NMR spectrum of *V. cholerae* O139 CPS (19, 35). The filtrate spectrum, in contrast, lacked chemical shifts for colitose, quinovosamine, GluNAc, and D-galactouronic acid (not shown). The retentate gave strong reaction with the murine *V. cholerae* O139 hyperimmune serum by Western blot and double immunodiffusion (not shown). The retentate, denoted CPS, showed only <0.5 endotoxin units/ μg as measured by the *Limulus* amebocyte lysate test.

AH derivatives of CPS (CPS_{AH}) and of rDT (rDT_{AH}). CP-S_{AH} contained 3.4% of AH, which represents ~ 1 AH per five CPS-repeating units (Table 1). CPS_{AH} and CPS formed a line of identity when reacted with murine hyperimmune *V. cholerae* O139 serum by double immunodiffusion (not shown).

rDT_{AH} contained 7.2 mol of AH per mol of protein and formed a line of identity with rDT when reacted with equine DT antiserum by double immunodiffusion (not shown).

Conjugates. (i) EDC-mediated synthesis of CPS_{AH}-rDT conjugates. Identical reaction conditions, except for the concentration of EDC, were used to prepare I:CPS_{AH}-rDT (0.05 M EDC) and II:CPS_{AH}-rDT (0.02 M EDC). Gel filtration of either conjugate on Sepharose CL-4B yielded three peaks at V_0 , K_d 0.4, and K_d 0.76 (Fig. 3A). Two peaks, at V_0 and K_d 0.4, consisted of both polysaccharide and protein, while the peak at K_d 0.76 contained only protein. Since the K_d s of the free CPS and rDT on Sepharose CL-4B are 0.4 and 0.76, respectively, the presence of unreacted CPS and/or rDT within the range of K_d 0.4 to 0.76 could not be excluded (Table 2). Accordingly, only the void volume fractions were pooled and are denoted I:CPS_{AH}-rDT and II:CPS_{AH}-rDT. I:CPS_{AH}-rDT had a lower polysaccharide/protein (wt/wt) ratio than did II:CPS_{AH}-rDT (0.46 $<$ 0.76). The yields of both conjugates were about 20% based upon the recovery of polysaccharide. Double immunodiffusion of either conjugate against murine *V. cholerae* O139 and equine DT toxin hyperimmune sera showed a single precipitin line (Fig. 4A).

(ii) CDAP-mediated synthesis of CPS-rDT_{AH} conjugates. I:CPS-rDT_{AH} and II:CPS-rDT_{AH} were prepared under the

same conditions except the CDAP/CPS ratios (wt/wt) were 4:5 and 1:5, respectively. Gel filtration of either conjugate on Sepharose CL-4B showed two peaks (V_0 and K_d 0.4), both containing polysaccharide and protein (Fig. 3B). The void volume fractions were pooled and are denoted as conjugates I:CPS-rDT_{AH} and II:CPS-rDT_{AH}; their polysaccharide/protein ratios were 0.90 and 0.99, respectively. The yield of I:CPS-rDT_{AH} was 45%. The yield of II:CPS-rDT_{AH} could not be determined because of an accidental loss of some material. Both conjugates formed a single precipitin line when reacted with murine *V. cholerae* O139 and equine DT hyperimmune sera by double immunodiffusion (Fig. 4B).

Serum antibody responses elicited by conjugates. (i) Anti-CPS IgG. CPS alone did not elicit a significantly different antibody response compared to saline (0.22 versus 0.19) (Table 3). None of the conjugates elicited a statistically significant antibody response after the first dose. All four conjugates elicited significant rises of anti-CPS IgG after the second dose ($P < 0.006$). However, only three, II:CPS_{AH}-rDT, I:CPS-rDT_{AH}, and II:CPS-rDT_{AH}, elicited a booster response following the third dose compared to the second one ($P < 0.003$). There were no significant differences between the post-third levels elicited by these three conjugates (10.3 versus 11.5 versus 4.21); all were significantly higher than those elicited by I:CPS_{AH}-rDT (10.3, 11.5, and 4.21 versus 0.43; $P < 0.0001$).

(ii) Anti-DT IgG. All conjugates elicited significant rises of anti-DT IgG after the second and third injections compared to

TABLE 2. Composition of *V. cholerae* O139 CPS conjugates with rDT

Conjugate	Conjugation method	PS/PR ratio (wt/wt)	% Yield ^a
I:CPS _{AH} -rDT	EDC (0.05 M)	0.46	28.0
II:CPS _{AH} -rDT	EDC (0.02 M)	0.78	20.1
I:CPS-rDT _{AH}	CDAP-CPS (4:5)	0.90	45.0
II:CPS-rDT _{AH}	CDAP-CPS (1:5)	0.99	—

^a Yield was determined based on the amount of polysaccharide in the conjugate. —, the accidental loss of some material prevented accurate determination of the yield.

FIG. 4. Double immunodiffusion of *V. cholerae* O139 conjugates with murine hyperimmune cholera O139 and equine DT antisera. (A) Representative pattern for CPS_{AH}-rDT conjugates. (B) Representative pattern for CPS-rDT_{AH} conjugates. Numbers: 1, murine hyperimmune cholera O139 antiserum, 3 μ l; 2, equine DT antiserum, 5 μ l; 3, conjugate (PS, 1 to 4 μ g; PR, 1 to 8 μ g).

the first injection ($P < 0.0001$). II:CPS_{AH}-rDT induced the highest level of anti-DT IgG of all conjugates; however, only the post-third injection level was statistically significantly higher (1,050 versus 245, 255, and 279; $P < 0.0007$).

Vibriocidal activity of murine sera. Eleven representative sera from mice injected three times with II:CPS_{AH}-rDT or I:CPS-rDT_{AH} were tested for vibriocidal activity (Table 4). Their titers ranged from 1,600 to 6,400: II:CPS_{AH}-rDT induced slightly higher titers (3,200 to 6,400) than did I:CPS-rDT_{AH} (1,600 to 3,200). These two groups of sera showed a similar range of anti-CPS IgG levels, while the anti-CPS IgM levels were slightly higher in mice injected with II:CPS_{AH}-rDT than in mice injected with I:CPS-rDT_{AH}. Similar vibriocidal results were demonstrated with the heavily capsulated *V. cholerae* O139 MDO12C variant (the strain which was used for

purification of the CPS) and other clinical isolates as the target strains (not shown).

Following treatment with 2-ME, the vibriocidal titers of most sera declined about fourfold; however, all retained significant levels of vibriocidal activity.

Vibriocidal activity in convalescent sera of cholera patients infected with *V. cholerae* O139. Vibriocidal titers of 20 patient sera ranged from 100 to 6,400 (Table 5). After absorption with CPS, titers of all sera declined to ≤ 50 (baseline for the assay).

Treatment with 2-ME reduced the vibriocidal activity to ≤ 50 in 17 of 20 sera. The vibriocidal titer of SK 639-2 remained at the same level (i.e., 400) as was found in the untreated serum.

DISCUSSION

Probably because of its complex structure (19, 35) and relatively tight folded conformation (10), the development of

TABLE 3. Serum IgG response specific to CPS and DT elicited in mice ($n = 10$ /group) by *V. cholerae* O139 CPS conjugates with rDT^a

Conjugate	Dose no.	EU/ml (25–75 centiles)	
		Anti-CPS IgG ^b	Anti-DT IgG ^c
I:CPS _{AH} -rDT	1	0.13 (0.11–0.17)	0.05 (0.03–0.06)
	2	0.35 (0.24–0.5)	52.8 (33.8–87.4)
	3	0.43 (0.28–0.58)	254 (121–376)
II:CPS _{AH} -rDT	1	0.23 (0.16–0.28)	0.60 (0.2–2.6)
	2	1.04 (0.35–0.68)	186 (155–233)
	3	10.3 (1.67–12.2)	1050 (715–1210)
I:CPS-rDT _{AH}	1	0.11 (0.11–0.14)	0.70 (0.37–1.3)
	2	0.89 (0.39–1.14)	81.1 (52.3–128)
	3	11.5 (4.99–29.7)	255 (128–548)
II:CPS-rDT _{AH}	1	0.22 (0.18–0.27)	0.80 (0.34–2.8)
	2	0.49 (0.35–0.68)	146 (90–214)
	3	4.21 (1.67–12.2)	279 (175–388)

^a CPS alone after three injections did not elicit anti-CPS IgG to a significantly different extent compared to saline (0.21 EU versus 0.19 EU).

^b All four conjugates elicited a significant rise of anti-Cls Ig after the second injection ($P < 0.006$). Only II:CPS_{AH}-rDT, I:CPS-rDT_{AH}, and II:CPS-rDT_{AH} elicited a booster after the third injection ($P < 0.003$).

^c All four conjugates elicited significant rises of anti-DT IgG after the second and third injections. After the third injection, II:CPS_{AH}-rDT elicited the highest and most statistically significantly different level compared to the other conjugates ($P < 0.0007$).

TABLE 4. Vibriocidal activity of representative sera from mice injected three times with the conjugates of *V. cholerae* O139 CPS and rDT^a

Conjugate	Amt of anti-CPS (EU)		Vibriocidal titer	
	IgG	IgM	Untreated serum	Treated with 2-ME
II:CPS _{AH} -rDT	13.2	5.43	3,200	400
	17.5	3.64	1,600	400
	42.2	6.94	3,200	800
	54.5	9.11	6,400	1,600
	180.8	10.5	>6,400	1,600
I:CPS-rDT _{AH}	15.5	4.02	1,600	400
	18.9	1.45	1,600	800
	27.2	2.05	1,600	200
	29.7	2.38	1,600	400
	36.3	2.54	3,200	800
	68.5	2.32	3,200	800

^a Serum anti-CPS IgG and IgM levels are expressed in EU/ml compared to a murine hyperimmune cholera O139 serum arbitrarily assigned 1,000 EU for anti-CPS IgG and 100 EU for anti-CPS IgM. The vibriocidal assay was performed with *V. cholerae* O139 isolate SPH1168 as the target strain and twofold serially diluted sera starting from a 1:50 dilution. The vibriocidal titer is defined as the reciprocal of the highest serum dilution that caused a $\geq 60\%$ reduction in the number of bacteria compared to the complement control. Sera from mice injected with saline or CPS had vibriocidal titer of < 50 .

TABLE 5. Serum vibriocidal titers of convalescent sera from patients infected with *V. cholerae* O139 measured before and after absorption with CPS or treatment with 2-ME

Patient	Vibriocidal titer ^a		
	Untreated	CPS absorbed	2-ME treated
SK 391-2	3,200	<50	<50
SK 395-2	1,600	<50	<50
SK 428-2	800	<50	<50
SK 456-2	400	<50	<50
SK 458-2	3,200	<50	<50
SK 494-2	100	<50	<50
SK 504-2	400	<50	<50
SK 522-2	1,600	<50	50
SK 577-2	1,600	<50	<50
SK 591-2	1,600	<50	<50
SK 597-2	800	<50	<50
SK 599-2	3,200	<50	<50
SK 622-2	400	<50	50
SK 639-2	400	<50	400
SK 646-2	3,200	<50	<50
SK 720-2	1,600	<50	<50
SK 741-2	800	<50	<50
SK 749-2	>6,400	50	100
SK 755-2	1,600	<50	200
SK 760-2	1,600	<50	50

^a Each vibriocidal assay was performed with twofold serially diluted tested serum starting from a 1:50 dilution and using *V. cholerae* O139 SPH1168 as the target strain.

synthetic schemes for preparation of *V. cholerae* O139 CPS conjugate vaccine was laborious and required using a readily available protein carrier such as chicken serum albumin (Kossaczka and Szu, submitted). Slight modifications of the two most successful synthetic schemes were used to prepare conjugates with the medically useful rDT. Both synthetic schemes involved ADH as the linker and two different activating agents (CDAP or EDC): (i) CDAP was used to prepare the AH derivative of CPS (CPS_{AH}) and EDC for rDT_{AH} and (ii) conjugation of CPS_{AH} with rDT (CPS_{AH}-rDT) was mediated by EDC, while CPS-rDT_{AH} conjugates were prepared by binding rDT_{AH} with CDAP-activated CPS. The resultant conjugates elicited serum anti-CPS IgG after the second injection and a booster response after the third injection when administered to mice by a clinically relevant method and route. Similar to the immunologic properties of the *V. cholerae* O1 Inaba O-specific polysaccharide conjugates with cholera toxin (11) and *V. cholerae* O139 CPS conjugates with chicken serum albumin (unpublished data), the *V. cholerae* O139 CPS-rDT conjugates elicited high titers of serum vibriocidal antibodies in mice. Treatment with 2-ME reduced (~4-fold) but did not eliminate their vibriocidal activity, indicating that much of this activity was mediated by anti-CPS IgG.

I:CPS-rDT_{AH} elicited the highest level of anti-CPS IgG after the third injection (11.4 EU), but this was not statistically different from the levels elicited by II:CPS_{AH}-rDT (10.3 EU) or II:CPS-rDT_{AH} (4.21 EU). On the basis of these data, we plan to clinically evaluate I:CPS-rDT_{AH} and II:CPS_{AH}-rDT.

All four conjugates elicited significant rises of anti-DT IgG after the second and third injections. II:CPS_{AH}-rDT elicited the highest post-third injection level of anti-DT IgG that was significantly different from those of the other three conjugates ($P < 0.0007$).

I:CPS_{AH}-rDT, prepared by synthesis of CPS_{AH} with rDT at the higher concentration of EDC (0.05 M), elicited the lowest level of anti-CPS IgG. The level of anti-DT IgG induced by this

conjugate was comparable to those elicited by both of CPS-rDT_{AH}, indicating that there was no correlation between the antibodies elicited to the CPS and those elicited to the protein carrier.

There is some confusion about the vibriocidal activity of convalescent sera from patients infected with *V. cholerae* O139 (3, 17, 25, 28, 40). We found that patient sera convalescent from cholera O139 were uniformly vibriocidal. The data variation among laboratories may be explained by the different complement dilutions used for the vibriocidal assays. We found that highly diluted complement, used in the vibriocidal assay for *V. cholerae* O1, is not sufficient to mediate killing of *V. cholerae* O139 which has a capsule. We showed that the undiluted baby rabbit serum, as the source of complement, is a reliable reagent for demonstrating antibody-initiated lysis of *V. cholerae* O139.

Similar to the serologic response of humans to the *V. cholerae* O1 infection (1, 24, 29, 32), our results showed that vibriocidal activity of sera from patients infected with serotype O139 was mostly specific to its surface polysaccharide (CPS) and mediated by IgM. This is also true for parenterally administered killed whole-cell cholera O1 vaccine or orally administered attenuated cholera O1 strains (7, 27, 44). In contrast, parenterally administered polysaccharide-protein conjugate vaccines elicit, in addition to IgM, high levels of serum anti-polysaccharide IgG (i.e., 2-ME resistant) (11, 38). We proposed that it is IgG that penetrates the intestinal epithelium and initiates complement-mediated lysis of the bacterial inoculum and that measurement of the conjugate-induced serum IgG specific to the surface polysaccharides of both *V. cholerae* O1 and O139 should provide a reliable method for standardization of these vaccine candidates (36, 39).

Diafiltration through YM100 allowed a rapid separation of the low-molecular-weight impurities from *V. cholerae* O139 CPS. When the material eluted at K_d 0.91 from Sepharose CL-4B, representing only 1% (by weight) of the unfractionated CPS, was concentrated 100 times and analyzed by SDS-PAGE and Western blot with murine hyperimmune cholera O139 antiserum, it showed two fast-moving bands (unpublished data), a finding similar to that reported for LPS of *V. cholerae* O139 (4, 45). Our results indicate that diafiltration could be adapted for rapid separation of LPS from other medically useful polysaccharides.

In summary, *V. cholerae* O139 CPS conjugates with rDT elicited high levels of serum anti-CPS IgG in mice with vibriocidal activity. The vibriocidal activity of convalescent sera from patients infected with *V. cholerae* O139 was mediated mostly by anti-CPS IgM. To verify whether a critical level of anti-CPS IgG will confer immunity to *V. cholerae* O139, clinical trials of the two most immunogenic CPS-rDT conjugates are planned.

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