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Synthesis, Characterization, and Some Immunological Properties of Conjugates Composed of the Detoxified Lipopolysaccharide of *Vibrio cholerae* O1 Serotype Inaba Bound to Cholera Toxin

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Protection against cholera has been correlated with the level of serum vibriocidal antibodies. The specificity of these vibriocidal antibodies was mostly to the lipopolysaccharide (LPS). We synthesized conjugates of detoxified LPS with cholera toxin (CT) and other proteins in order to elicit serum LPS antibodies with vibriocidal activity. Treatment with hydrazine (deacylated LPS) reduced the endotoxic properties of the LPS to clinically acceptable levels and resulted in a molecule larger and more antigenic than the saccharide produced by acid hydrolysis. More immunogenic conjugates resulted from multipoint compared with single-point attachment of the deacylated LPS to the protein. The conjugates containing CT had low levels of pyrogen and no toxic activity upon Chinese hamster ovary cells and elicited booster responses of vibriocidal and CT antibodies when injected subcutaneously as saline solutions into mice; the vibriocidal titers were similar to those elicited by comparable doses of cellular vaccines. We suggest how serum vibriocidal antibodies might prevent cholera.

Unhappily, cholera persists as a cause of illness and death in at least 40 countries on three continents; ~340,000 cases have been reported in the Western Hemisphere since an epidemic started in Peru in January 1991 (16, 32). Worldwide prevention of cholera by immunization has not been achieved because of the limitations of available vaccines. Research into new vaccines is difficult because there is no consensus about the identities of the human protective moieties and because animal models provide incomplete information about the nature of human protective immunity to cholera (20, 21, 25). The absence of bacterial invasion, systemic signs, and intestinal inflammation has led to the understanding that cholera is a toxin-mediated disease of the luminal surface of the jejunum and to the notion that a local intestinal response is required for protective immunity (4, 10–12, 20, 21, 25, 26, 33, 35, 46, 50).

The lipopolysaccharide (LPS) of *Vibrio cholerae* is considered to be a protective antigen (3, 18, 21, 35, 38, 41, 44, 52, 61), but the structures, pathogenic role, and host moieties involved in protective immunity to cholera are incompletely understood. *V. cholerae* O1 LPS contains lipid A and a core oligosaccharide composed of 4-amino-4-deoxy-L-arabinose, quinovosamine, D-glucose, D-fructose, and heptose (23, 30, 48). KDO (3-deoxy-D-manno-octulosonic acid) has been identified and presumed to be in the core adjacent to the lipid A (5). The O-specific polysaccharide (O-SP) of *V. cholerae* O1, serotype Inaba, contains a saccharide of ~12 residues composed of 1→2-linked D-perosamine whose amino groups are acylated by 3-deoxy-L-glycero-tetronic acid (23, 30, 48). The structures of the three domains of *V. cholerae* LPS have not been related to the serological specificity of the serotypes (LPS types) Inaba and Ogawa (23, 35, 40).

Parenterally administered cellular vaccines or partially purified LPS induced a statistically significant serotype-specific protection (~60%) in adults for ~6 months (3, 7, 18, 21, 38, 45). Cellular vaccines were less effective for infants and young children and ineffective for control of outbreaks of cholera (38, 51). The protective immune component induced by these vaccines was proposed to be serum LPS antibodies with vibriocidal activity (1, 3, 18, 21, 38, 41). Cellular cholera vaccines did not elicit serum antitoxin (37) or, by analogy with similar products, secretory antibodies (57). Similar effects were also obtained with orally administered inactivated *V. cholerae* (7, 10–12): addition of the B subunit of cholera toxin (CT) did not recruit additional protection (11). Although considered as a marker and not as a protective moiety, vibriocidal antibody levels are a reliable method for predicting resistance to cholera (3, 4, 7, 12, 18, 21, 38, 39). Our interpretation of these data is that cellular cholera vaccines, as observed with similar products and polysaccharides, were poor immunogens and had T-cell-independent properties (14, 38, 39, 50). To solve the problems of adverse reactions, the lesser immunogenicity in infants and young children, and the T-cell-independent properties of the LPS in cellular vaccines, we synthesized conjugates, composed of detoxified LPS of *V. cholerae* serotype Inaba, to several proteins, including CT (49). The synthesis and immunological properties of these investigational vaccines in mice, using CT as a carrier, are described.

MATERIALS AND METHODS

Bacterial strains. *V. cholerae* 569B (biotype classical, serotype Inaba), which produces CT-1 (29), and NIH 41 (biotype classical, serotype Ogawa) were used for the vibriocidal assay. *V. cholerae* 2524 (biotype classical, serotype Inaba) (Katherine Greene, Centers for Disease Control,

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Atlanta, Ga.) was used for raising LPS antiserum in mice. *V. cholerae* O75 (serotype Inaba, biotype El Tor), which produces CT-2 (29), was a recent isolate from South America (Richard Haberberger, Naval Medical Research Institute, Bethesda, Md.).

Reagents. Anhydrous hydrazine, adipic acid dihyrazide (ADH), dithiothreitol, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC), disodium EDTA, KDO, RNase, DNase, and pronase were from Sigma Chemical Co., St. Louis, Mo. *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and deoxycholic acid were from Calbiochem, La Jolla, Calif. *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), alum, 2,4,6-trinitrobenzesulfonic acid (TNBS), and BCA reagent for protein determination were from Pierce Chemical Co., Rockford, Ill. CNBr was from Eastman Chemical, Rochester, N.Y. Sephadex G-25, Sephacryl S-300, Superose 12 column (10 by 300 mm), and dextrans for molecular weight assays were from Pharmacia-LKB, Piscataway, N.J. LPS from *V. cholerae* 569B (Inaba) was from List Biologicals, Campbell, Calif. *Limulus* amoebocyte lysate (LAL) was from Associates of Cape Cod, Woods Hole, Mass. *p*-Nitrophenyl phosphate was from Fluka, Ronkonkoma, N.Y. The U.S. standard for endotoxin was a gift from Donald Hochstein, Food and Drug Administration, Bethesda, Md.

Analytic methods. The molecular sizes of LPS, O-SP, and deacylated LPS (DeA-LPS) were estimated by gel filtration through Superose 12 in 0.2 M NaCl–1 mM EDTA–10 mM Tris–0.25% deoxycholic acid (pH 8.0), using the dextran standards. KDO was measured by the thiobarbituric acid assay, using KDO as a standard (5). The extent of derivatization of DeA-LPS with adipic acid hydrazide was measured by TNBS, using ADH as a standard (9). Protein was measured by the BCA reagent, using bovine serum albumin as a standard. Hexose was measured by the anthrone reaction, using O-SP as a standard (55). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for detection of LPS (56). Data for nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GSX-500 spectrometer. Each spectrum was acquired with broad-band ^1H decoupling at 90°C 10-ms carbon-observed pulse, 32K data points which were zero filled to 64K points prior to Fourier transformation, 30-KHz spectral window (0.54-s acquisition time), and 3.0-s delay between pulse cycles. Prior to Fourier transformation, each free-induction-decay signal was exponentially multiplied so as to result in an additional 4-Hz line broadening in the frequency domain spectrum. Approximately 10 mg of each sample was dissolved in 0.5 ml of D_2O and recorded at ambient probe temperature (21°C).

Proteins. CT-1 lot 582 (Pasteur Merieux Serums & Vaccins, Lyon, France) and CT-1 lot rst were purified from *V. cholerae* Inaba strain 569B (20, 29). Anti-mouse immunoglobulin G (IgG) and IgM alkaline phosphatase conjugates were from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.

Polysaccharides. LPS was detoxified by two methods. For acid hydrolysis, LPS (10 mg/ml in 1% acetic acid) was heated at 100°C for 90 min (60). The reaction mixture was ultracentrifuged at 60,000 × *g* at 10°C for 5 h, dialyzed exhaustively against H_2O at 3 to 8°C, passed through a sterile 0.20-μm-pore-size filter (Nalge, Rochester, N.Y.), and freeze-dried (designated O-SP). For hydrazine hydrolysis, LPS (10 mg/ml) was treated with hydrazine at 37°C for 2 h (hydrazine treatment has been reported to remove esterified fatty acids from lipid A; accordingly, this product is designated DeA-LPS) (34). This material was mixed with acetone in an ice bath until a precipitate formed (~90% acetone), and the reaction mixture was centrifuged at 15,000 × *g* at 10°C for 30 min. The precipitate was washed two times with acetone and dissolved in 0.15 M NaCl (pH 7.0) to ~3 mg/ml. The reaction mixture was centrifuged at 60,000 × *g* for 5 h at 10°C; the supernatant was dialyzed exhaustively against H_2O, passed through a 0.22-μm-pore-size filter, and freeze-dried. The protein and nucleic acid concentrations of O-SP and DeA-LPS were <1%. LPS, extracted from acetone-dried cells of El Tor biotype Ogawa serotype strain 3083-13, was used for inhibition of vibriocidal activity.

Conjugation of DeA-LPS with proteins. Two schemes were used. Method 1 utilized SPDP to thiolate both the protein and the terminal amino group on the core of DeA-LPS as described for the cell wall polysaccharide of pneumococci (54). This scheme will give single-point attachment between DeA-LPS and protein. DeA-LPS (3 mg/ml) or protein (10 mg/ml) was dissolved in 0.15 M HEPES–2 mM EDTA (pH 7.5). SPDP (20 mM in ethanol) was added dropwise at weight ratios of 0.5 for DeA-LPS and 0.2 for protein. The reaction mixture was stirred at ambient temperature for 1 h and passed through a Sephadex G-25 column (5 by 35 cm) in H_2O for DeA-LPS-SPDP and in phosphate-buffered saline (PBS) for protein. The DeA-LPS-SPDP derivative was freeze-dried, and the protein was concentrated by membrane filtration (Amicon YM10 filter). The derivatization with SPDP in aliquots of DeA-LPS or the proteins was determined by reduction of the *N*-pyridyl disulfide bond with 40 mM dithiothreitol and assuming a molar extinction coefficient at A_{340} of 8.08×10^4 (53). The *N*-pyridyl disulfide on DeA-LPS-SPDP was reduced with 40 mM dithiothreitol and passed through a column (2.5 by 50 cm) of Sephadex G-25 in 0.2 M NaCl, and the void volume fractions were mixed with the SPDP derivative of the protein. This reaction mixture was stirred at room temperature for 2 h and passed through a column (5 by 100 cm) of Sephacryl S-300 in 0.2 M NaCl, and the void volume fractions were pooled. The conjugate synthesized by this method was designated DeA-LPS-CT_I.

An aliquot of DeA-LPS-CT_I in saline was treated with 0.05 M EDAC at room temperature for 1 h at pH 6.0 to cross-link the conjugate. The nonreacted EDAC was removed by exhaustive dialysis.

In method 2, DeA-LPS was derivatized with ADH as described for *Haemophilus influenzae* type b and *Shigella dysenteriae* type 1 O-SP (9, 49). This synthetic method will produce multiple sites of attachment between the saccharide and protein. DeA-LPS (10 mg/ml saline) was brought to pH 10.5 with 1 N NaOH, and an equal weight of CNBr (1 g/ml of acetonitrile) was added. The pH was maintained between 10.0 and 11.0 with 1 N NaOH for 3 min. An equal volume of 0.5 M ADH in 0.5 M $NaHCO_3$ was added, and the pH was adjusted to 8.5. The reaction mixture was stirred at room temperature for 1 h and then at 3 to 8°C overnight and passed through a Sephadex G-25 column (5 by 35 cm) in H_2O. Fractions from the void volume were pooled and freeze-dried. The DeA-LPS-ADH derivative was dissolved in 0.15 M NaCl to 10 mg/ml. An equal volume of protein (~10 mg/ml) was added, and the pH was adjusted to 5.5 with 0.1 M HCl. EDAC was added to a final concentration of 0.05 M, and the pH maintained at 5.5 to 6.0 for 1 h. The reaction mixture was passed through a column (2.5 by 90 cm) of Sephacryl S-300 in 0.2 M NaCl, and the fractions in the void volume were pooled. Conjugates synthesized by using CT lots 582 and rst as carriers are designated DeA-LPS-CT_{II} and DeA-LPS-CT_{III}, respectively.

Biological assays. LPS concentration, assayed by the LAL assay, was expressed in endotoxin units (EU) related to the U.S. standard (24). Pyrogenicity in rabbits was kindly assayed by Donald Hochstein, Food and Drug Administration. The toxicity of CT was estimated by Chinese hamster ovary (CHO) cell elongation (15).

Complement-mediated vibriocidal antibody was measured against Inaba and Ogawa strains (19, 22). Tenfold serum dilutions were mixed with equal volumes of ~1,000 cells per ml in diluted guinea pig serum and incubated at 37°C for 1 h. A hyperimmune serum was used as a standard in each assay. The titer was expressed as the reciprocal of the highest dilution of serum that yielded 50% vibriocidal activity. Some sera were assayed for vibriocidal antibodies against strains 569B and O75 of serotype Inaba; the titers of these sera were identical against both strains. Therefore, vibriocidal activities of the sera were assayed with strain 569B. Inhibition of vibriocidal activity was assayed by mixing 100 μg of LPS, DeA-LPS, O-SP, or CT per ml with various dilutions of antisera at 37°C for 1 h prior to addition of the bacteria (19).

Immunization. Hyperimmune LPS antiserum was prepared by injecting female adult BALB/c mice with heat-killed *V. cholerae* 2524 (42). Burro CT antiserum was prepared as described previously (13). For evaluation of immunogenicity, 6-week-old BALB/c or general-purpose mice (National Institutes of Health [NIH], Bethesda, Md.) were injected subcutaneously with 2.5 or 10 μg of DeA-LPS alone or as a conjugate in saline. Mice were injected at 2-week intervals and bled 7 days after each immunization. The fourth dose was given 4 weeks after the third injection, and the mice were bled 7 days and 6 months later. Groups of mice were immunized similarly with conjugates adsorbed with 0.125 or 1.25 mg of aluminum hydroxide per dose. Cellular cholera vaccine (Frank McCarthy, Wyeth-Ayerst Laboratories, Marietta, Pa.) containing 4×10^9 each of Inaba and Ogawa serotypes was used as a control. Mice were immunized with 0.1 or 0.2 ml of the vaccine.

Serological assays. Double immunodiffusion was performed in 1% agarose in PBS. LPS and protein antibody levels were determined by enzyme-linked immunosorbent assay (ELISA), using Immunolon 4 plates (Dynatech, Chantilly, Va.). Each well of the plates was coated with 100 μl of either LPS (10 μg/ml) or CT (5 μg/ml) in PBS. LPS antibody levels were expressed in ELISA units, using hyperimmune sera as a reference. CT antibody levels were expressed in ELISA units with a hyperimmune mouse pooled standard serum prepared by repeated immunization of mice with CT (Douglas Watson, NIH).

Statistical analyses. Antibody levels are expressed as the geometric mean. Antibody concentrations below the sensitivity of the ELISA were assigned values of one-half of that level. Comparison of geometric means was performed with the two-sided *t* test and the Wilcoxon test.

RESULTS

Characterization of LPS, DeA-LPS, and O-SP. Silver-stained SDS-PAGE of 2.5 μg of LPS from Inaba showed two faint bands with a ladder in the middle and two dense bands near the bottom of the gel (Fig. 1). Typical ladders of higher-molecular-weight O-SP were formed by the LPS from *Escherichia coli* O111. No bands were observed with 10-μg samples of either O-SP or DeA-LPS. By the LAL assay, the LPS of serotype Inaba had 3×10^3 to 6×10^3 EU/μg and the DeA-LPS had 3 EU/μg; this level represented a ~1,000-fold reduction.

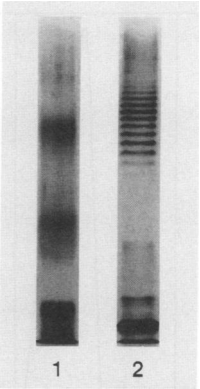

FIG. 1. Silver-stained SDS-PAGE in 14% gel of 2.5 μg of LPS from *V. cholerae* serotype Inaba (lane 1) and *E. coli* O111 (lane 2).

Immunodiffusion showed a single band of precipitation between the LPS and the hyperimmune LPS sera (Fig. 2a). A more diffuse band was observed with DeA-LPS, which yielded a partial identity reaction with LPS. Neither O-SP nor CT precipitated with this hyperimmune serum. The molecular sizes of LPS, O-SP, and DeA-LPS were estimated by high-pressure liquid chromatography (HPLC) on Superose 12 (Fig. 3). LPS and DeA-LPS showed two peaks: LPS had K_d values of 0.40 (16,000 Da) and 0.46 (8,700 Da), and DeA-LPS had K_d values of 0.38 (13,000 Da) and 0.50 (6,000 Da). O-SP exhibited only one peak corresponding to the second peak of DeA-LPS (K_d 0.51, 5,900 Da). Because of its greater antigenicity and high molecular weight, DeA-LPS was used as the saccharide for the conjugates. As described, we could not detect KDO in either O-SP or DeA-LPS by the thiobarbituric acid assay (5, 58).

The ^{13}C NMR spectra of DeA-LPS and O-SP were in agreement with previous reports (30, 48). Each spectrum showed 10 major signals with chemical shifts identical, or nearly identical, to those reported (Fig. 4).

Characterization of conjugates. The extents of derivatization of DeA-LPS with SPDP (1.18%) and with ADH (1.76%) were similar (Table 1). The DeA-LPS/protein (wt/wt) ratios were slightly lower for the conjugates of CT prepared with SPDP than with ADH, ranging from 0.72 for DeA-LPS-CT_I to 1.5 for DeA-LPS-CT_{III}. The yields for all of the conjugates were ~80%, as calculated by the recovery of saccharide in the conjugates compared with the derivatized polysaccharide. Similar results were obtained by method 2 with tetanus toxoid as the carrier (data not shown).

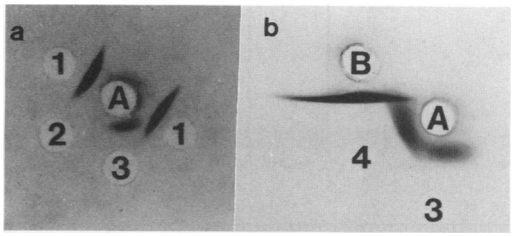

FIG. 2. Double immunodiffusion. (a) A, Hyperimmune *V. cholerae* serotype Inaba serum. Outer wells: 1, Inaba LPS, 250 μg/ml; 2, Inaba O-SP, 250 μg/ml; 3, Inaba DeA-LPS, 250 μg/ml. (b) A, Hyperimmune *V. cholerae* serotype Inaba serum; B, hyperimmune CT antiserum. Outer wells: 3, Inaba DeA-LPS, 250 μg/ml; 4, conjugate DeA-LPS-CT_{II}.

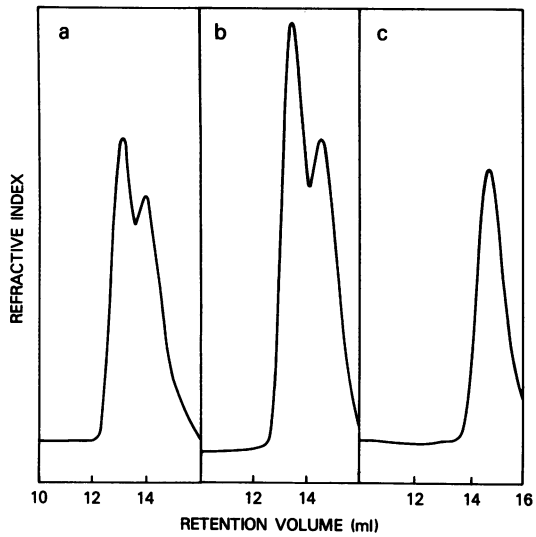

FIG. 3. HPLC profiles of 100-μl samples (1.0 mg/ml) through a column (10 by 300 mm) of Superose 12 in 0.2 M NaCl–0.01 M Tris–0.001 M EDTA–0.25% deoxycholic acid (pH 8). (a) LPS serotype Inaba; (b) DeA-LPS Inaba; (c) O-SP Inaba.

A double-immunodiffusion experiment showed that the serotype Inaba hyperimmune antiserum yielded an identical line of precipitation with the DeA-LPS and DeA-LPS-CT_{II} (Fig. 2). Similarly, the CT and LPS antisera yielded a line of identity with DeA-LPS-CT_{II} and CT. A faint spur from the CT antiserum extended over the LPS antiserum and the conjugate, suggesting there was a slight amount of unbound CT antigen in this preparation.

The residual toxicity of CT and DeA-LPS in the conjugates was estimated by in vitro and in vivo assays. In the thermal induction test, DeA-LPS was not pyrogenic when injected at 1 μg/kg of rabbit body weight. The endotoxin content of the conjugates was ~2 EU/μg by the LAL assay. CT induced elongation in CHO cells at 0.4 ng/ml. The amount of CT, as a conjugate required to elicit the same degree of elongation, was 10^3- to 10^{10}-fold greater. DeA-LPS-CT_{III} had no detectable toxicity in the CHO cell assay

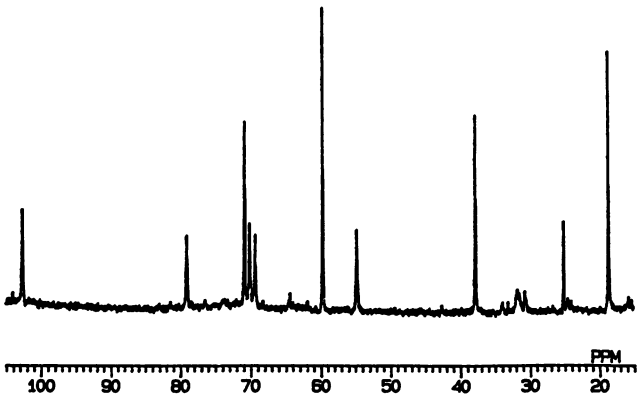

FIG. 4. ^{13}C NMR spectrum of the DeA-LPS from *V. cholerae* serotype Inaba. The 10 major signals were identical to those reported by Kenne et al. (30). The ^{13}C NMR spectrum of the acid-treated LPS (O-SP) was almost identical to this spectrum (not shown).

TABLE 1. Characterization of *V. cholerae* DeA-LPS-protein conjugates[a]

Conjugate	Linker	Linker/DeA-LPS (wt/wt)	SPDP/protein (wt/wt)	Yield (%)	DeA-LPS/protein (wt/wt)	Composition (mg/ml)	
						Protein	CHO
DeA-LPS-CT_I	SPDP	1.18	8.8	88	0.72	2.0	1.44
DeA-LPS-CT_I[b]	SPDP	1.18	8.8	NA[c]	0.65	1.15	0.75
DeA-LPS-CT_{II}	ADH	1.76	NA	79	0.80	0.48	0.38
DeA-LPS-CT_{III}	ADH	1.76	NA	88	1.50	1.0	1.50

[a] Polysaccharide was measured by anthrone reaction with DeA-LPS as a standard (55). The yield was calculated on the basis of the weight of the saccharide in the conjugate compared with the starting weight of the adipic acid hydrazide derivative. CHO, carbohydrate.
[b] Further treated with 0.05 M EDAC (Materials and Methods)
[c] NA, not applicable.

at 1.0 mg/ml and passed the general safety test in guinea pigs at 10 human doses (25 μg of DeA-LPS per dose) as described in the *Code of Federal Regulations* C, 610.13(b).

LPS antibodies (ELISA). There were no detectable LPS antibodies in either general-purpose or BALB/c mice immunized with 2.5 or 10 μg of DeA-LPS alone after any injection (data not shown). Table 2 shows the levels of antibodies to LPS in general-purpose mice immunized with DeA-LPS-CT_I and DeA-LPS-CT_{II}. Neither conjugate elicited LPS antibodies after the first immunization. DeA-LPS-CT_{II} elicited IgG and IgM antibodies after the second injection. Both conjugates elicited a significant rise of IgG antibodies after the third and fourth injections ($P < 0.01$). The IgG levels after the fourth injection were similar in mice injected with either the LPS or DeA-LPS-CT_{II}; LPS doses of 2.5 or 10.0 μg elicited IgG antibodies only after the third injection. The IgG levels were similar in the sera taken 7 days or 6 months after the fourth injection of DeA-LPS-CT_{II}. Similar levels of antibodies were elicited by 10-μg doses of the conjugates, by EDAC treated DeA-LPS-CT_I, and by conjugates adsorbed onto alum (data not shown).

Table 3 shows that the LPS antibody levels elicited by

TABLE 2. Serum IgM and IgG LPS antibodies (ELISA) elicited in general-purpose mice immunized with DeA-LPS alone or as a conjugate[a]

Vaccine	Dose (μg)	Injection no.	*n*	Geometric mean (25th–75th centiles)	
				IgG[b]	IgM
LPS	2.5	2	10	<10	320
		3	9	320	640
	10.0	2	3	<10	50 (10–160)
		3	10	149[j] (5–2,153)	260 (40–1,522)
		4	10	1,085[d] (80–12,800)	1,525 (269–5,120)
DeA-LPS-CT_I	2.5	2	5	<10	<10
		3	8	22[e] (5–95)	11 (5–24)
		4	5	318[f] (7–20,239)	152 (10–2,263)
DeA-LPS-CT_{II}	2.5	2	5	35 (14–80)	23 (14–40)
		3	11	80[g] (20–320)	150 (20–640)
		4	11	1,540[h] (320–12,800)	219 (40–640)
		4[c]	5	640[i] (453–905)	80 (40–226)

[a] Female general-purpose mice, ~6 weeks old, were injected subcutaneously with saline solutions of the antigen every 2 weeks for three times and then were given a fourth injection 4 weeks later.
[b] h versus f, d, and i, *P* not significant; h versus g, $P = 0.002$; d versus j, $P = 0.08$; f versus e, $P = 0.06$.
[c] The mice were bled 7 days after each injection and then again 6 months after the fourth injection.

TABLE 3. Serum IgM and IgG anti-LPS antibodies elicited in BALB/c mice immunized with DeA-LPS-CT conjugates or cellular cholera vaccine

Vaccine	Dose	Injection no.	n	Geometric mean (25th–75th centiles)	
				IgG[b]	IgM
DeA-LPS-CT_I	2.5 μg	1	5	<10	40 (40–40)
		2	5	<10	40 (40–40)
		3	10	<10	53 (40–40)
		3[a]	10	13 (5–24)[f]	65 (40–160)
DeA-LPS-CT_{II}	2.5 μg	1	5	<10	30 (20–40)
		2	5	<10	70 (40–160)
		3	10	46 (5–761)	130 (40–640)
		3[a]	6	32 (10–95)[g]	50 (40–80)
Whole cell	0.1 ml	1	5	<10	106 (57–226)
		2	5	139 (40–640)[c]	1,114 (453–2,560)
		3	9	1,742 (905–2,560)[d]	742 (400–1,600)
		3[a]	6	90 (67–160)[e]	101 (67–190)

[a] Mice bled 5 months after the third immunization.
[b] e versus f, $P = 0.0004$; e versus g, P = not significant; e versus d, $P = 0.0001$; d versus c, $P = 0.02$.

conjugates in BALB/c mice were lower than those of the general-purpose mice. After the first and second doses, there were low levels of IgM and no detectable IgG antibodies in BALB/c mice injected with the conjugates; low levels were detected after the third injection. The antibody levels remained similar 5 months after the last injection. The cellular vaccine induced high levels of both IgG and IgM after the second injection and a booster effect on the IgG antibody levels following the third injection; doses of 0.1 and 0.2 ml elicited similar levels (not shown). The IgG levels of the mice injected with the cellular vaccine declined to ~1/20 of their optimal values 5 months after the last injection ($P = 0.0001$) and were similar to those elicited by DeA-LPS-CT_{II}.

Vibriocidal antibodies. Neither 2.5 nor 10 μg of DeA-LPS or PBS (controls) elicited vibriocidal antibodies to *V. cholerae* serotype Inaba or Ogawa (not shown).

DeA-LPS-CT_{II} elicited low levels of vibriocidal antibodies to the Inaba serotype in general-purpose mice after the first injection (Table 4). Both DeA-LPS-CT_I and DeA-LPS-CT_{II} elicited booster responses after the next two injections. LPS elicited the highest level of vibriocidal antibodies.

In BALB/c mice, both conjugates elicited vibriocidal antibodies after the first injection; only DeA-LPS-CT_{II} elicited booster responses following the second and third injections (Table 5). The conjugates elicited higher vibriocidal activity to the homologous serotype (Inaba) than to the heterologous serotype (Ogawa). The cellular vaccine, which contains both serotypes, induced higher levels of vibriocidal antibodies against Ogawa than Inaba. The vibriocidal levels to serotype Inaba were elicited earlier and in higher titer by the cellular vaccine than by the conjugates. After the third injection, the vibriocidal levels to Inaba elicited by the whole cell and conjugate vaccines were similar.

All vibriocidal activity was removed from the conjugate-induced antibodies following absorption with either LPS, DeA-LPS, or O-SP of the Inaba serotype. Absorption with the Inaba LPS also removed all of the vibriocidal activity from the sera of mice injected with the cellular vaccine. DeA-LPS and O-SP, in contrast, removed ~90% of the vibriocidal activity from these sera. Absorption with the Ogawa LPS removed ~90% of the vibriocidal activity against serotype Inaba. Absorption with CT did not change the vibriocidal titers from the sera of mice injected with either the conjugates or the cellular vaccines.

Adsorption of DeA-LPS-CT_I, DeA-LPS-CT_{II}, or other conjugates onto alum had no effect on their immunogenicity (not shown).

CT antibodies. Significant rises of CT antibodies were elicited in all mice of both strains by both conjugates after each injection (Table 6).

TABLE 5. Vibriocidal activities of sera from BALB/c mice immunized with DeA-LPS alone or conjugated to CT or with whole cell cholera vaccine

Vaccine	Dose	Target	Reciprocal vibriocidal titer[a]		
			1st injection	2nd injection	3rd injection
DeA-LPS-CT_I	2.5 μg	Inaba	100	100	5,000
		Ogawa	25	100	1,000
DeA-LPS-CT_{II}	2.5 μg	Inaba	250	5,000	100,000
		Ogawa	100	500	50,000
Whole cell	0.1 ml	Inaba	2,500	50,000	100,000
		Ogawa	25,000	500,000	1,000,000

[a] Vibriocidal antibody titer of pooled sera after each dose.

DISCUSSION

Parenterally injected inactivated cellular vaccines as well as LPS elicited protective immunity, albeit at less than optimal levels and of limited duration, to cholera (3, 7, 8, 18, 21, 38, 46). It is likely that the protective mechanism elicited

TABLE 4. Vibriocidal antibody titers of pooled sera from NIH general-purpose mice immunized with conjugates or LPS[a]

Immunogen	Dose (μg)	Challenge serotype	Titer at immunization no.:		
			1	2	3
LPS	10.0	Inaba	ND[b]	50,000	500,000
DeA-LPS-CT_I	2.5	Inaba	ND	500	50,000
	2.5	Ogawa	ND	100	50,000
DeA-LPS-CT_{II}	2.5	Inaba	100	25,000	50,000
	2.5	Ogawa	<10	1,000	25,000
	10.0	Inaba	500	25,000	100,000
	10.0	Ogawa	50	1,000	50,000

[a] General-purpose mice from NIH were injected subcutaneously with 2.5 μg of DeA-LPS as a conjugate, and their sera were pooled in equal amounts for each group.
[b] ND, not done.

TABLE 6. Serum IgG CT antibodies in mice immunized with DeA-LPS conjugates[a]

Vaccine	Injection no.	Geometric mean (25th–75th centiles)[b]	
		BALB/c mice	General-purpose mice
DeA-LPS-CT_I	1	0.1[m] (0.1–0.2)	0.1[f] (0.1–0.2)
	2	56.9[n] (44–76)	49.0[g] (37–69)
	3	217.4[n] (152–270)	157.1[h] (134–198)
DeA-LPS-CT_{II}	1	0.1[c] (0.1–0.2)	0.1[i] (0.03–0.3)
	2	30.6[d] (11–80)	17.8[j] (11–31)
	3	136.7[e] (102–198)	156.0[k] (105–201)

[a] Mice immunized with whole cell cholera vaccine, DeA-LPS, or PBS had <0.01 ELISA antibody levels.
[b] n versus m, P = <0.001; d versus c, $P = 0.0001$; e versus d, $P = 0.04$; h and g versus f, $P < 0.01$; b versus e, $P = 0.01$; j and k versus i, $P < 0.01$.

by these two types of vaccines is serum vibriocidal antibodies with LPS specificity (little or no secretory antibodies are elicited by cellular vaccines or LPS) (3, 12, 18, 19, 57). Serum vibriocidal activity was also correlated with resistance against disease following convalescence from cholera by administration of live attenuated strains or of inactivated *V. cholerae* alone or with the B subunit of CT (4, 10, 12, 21, 38, 39, 51). Lastly, the age-related acquisition of vibriocidal antibodies in endemic areas parallels the increasing resistance to cholera observed in older children and adults (1, 38, 39). On the basis of these observations, we developed detoxified LPS-protein conjugate vaccines in order to elicit a vibriocidal antibody response with T-cell-dependent properties (49).

The preparation of conjugates to elicit LPS antibodies is difficult because (i) the complete structures of the LPS of the two serotypes are not known and (ii) the size of the Inaba O-SP is relatively small (~6,000 Da) (the immunogenicity of saccharides alone or in conjugates is directly related to their size) (2, 17, 53). Acidic hydrolysis of LPS from strain 569B (Inaba) resulted in a product of ~5,900 Da that had clinically acceptable levels of endotoxin but which did not precipitate with hyperimmune sera. Treatment with hydrazine produced LPS that was detoxified to acceptable levels, had molecular sizes of ~13,000 and 6,000 Da, and retained its antigenicity. Accordingly, the hydrazine-treated LPS was used to prepare the conjugates. As demonstrated for O-SP of *S. dysenteriae* type 1 (9), conjugates prepared by multipoint attachment (DeA-LPS-CT_{II}) elicited higher levels of LPS antibodies than did those prepared by single-point attachment (DeA-LPS-CT_{I}).

The conjugates described here, injected subcutaneously in saline at 1/10 the proposed human dose, elicited LPS antibodies with vibriocidal activity in young outbred mice. This immunization scheme in mice was predictive of the immunogenicity of *H. influenzae* type b-tetanus toxoid conjugates in infants injected concurrently with diphtheria-tetanus-pertussis vaccine (49). The low levels of endotoxic activity, as measured by the LAL and rabbit pyrogen assays, provide assurance that our conjugates will elicit little or no adverse reactions encountered with cellular vaccines for cholera (24).

On the basis of similar reasoning, Kabir synthesized a bivalent conjugate composed of NaOH-treated LPS from serotypes Inaba and Ogawa bound to a protein extract of *V. cholerae* 395 (Ogawa) (27). One milligram of this conjugate, in complete Freund's adjuvant, elicited antibodies in rabbits with vibriocidal activity against the two serotypes. The route of immunization, using complete Freund's adjuvant, and the dosage used are, however, clinically unacceptable.

None of our conjugates elicited IgG antibodies following the first injection. This apparent lesser immunogenicity of DeA-LPS-CT conjugates than of those with capsular polysaccharides (49) could be due to two factors: (i) the lesser immunogenicity of the O-SP of *V. cholerae* O1 as a result of its simplicity (linear homopolymer of perosamine acylated by 4-amino-4,6-dideoxy-L-glycero-tetronic acid) (30, 48) (ii) the relatively low molecular weight of the O-SP of *V. cholerae* O1 (35, 47). We plan to study the effect of cultivating *V. cholerae* at lower temperatures to increase the length of the LPS or to prepare polymeric forms of the DeA-LPS which could increase the immunogenicity of our conjugates (36).

In 6- to 12-month-old infants but not in older children or adults, cellular vaccines elicited booster responses to the saccharide component (14, 31). In mice, cellular vaccines elicited booster responses of LPS and of vibriocidal antibodies. This apparent difference may be due to preexisting antibodies in humans, probably stimulated by *V. cholerae* or cross-reacting antigens (1, 22, 40); these antibodies were not detected in the preimmune sera of the mice. The vibriocidal antibody response in older humans elicited by cellular vaccines could, therefore, represent a booster response. In mice, cellular vaccines and DeA-LPS-CT_{II} elicited similar levels of vibriocidal antibodies to serotype Inaba; this activity could be absorbed with LPS or DeA-LPS. Both types of vaccines elicited antibodies to serotype Ogawa although, as expected, the cellular vaccine, which contains organisms of both serotypes, elicited higher antibody levels to serotype Ogawa.

How could serum vibriocidal antibodies prevent cholera, which is caused by a noninvasive organism, whose symptoms are mediated by an exotoxin, and which is not accompanied by inflammation? First, serum antibodies, especially those of the IgG class, penetrate into the lumen of the intestine (28, 59); it is likely complement proteins are also present. Second, the walls of the intestine are in contact due to peristalsis. Third, the inoculum that survives the gastric acid is probably low or ~10^3 *V. cholerae* (22, 33). Fourth, *V. cholerae* organisms have short polysaccharides on their LPS; this trait is associated with a high susceptibility to the complement-dependent action of serum antibodies (43). These factors, namely, low inocula, serum vibriocidal antibodies, and complement molecules at the mucosal surface whose surfaces are pressed upon each other and churning a susceptible organism, provide an explanation for how serum vibriocidal antibodies confer protection against cholera: ingested *V. cholerae* are lysed on the intestinal mucosal surface.

Although the use of another component from *V. cholerae* may obscure the nature of protection elicited by our conjugates, we chose CT because it served as an immunogenic carrier for both the *H. influenzae* type b and the Vi polysaccharides (49, 53). Further, there remains the possibility that serum antitoxin, specific for the CT of the infecting strain, is protective or exerts synergistic protective activity with LPS antibodies (44, 52). The development of a nontoxic genetically engineered mutant protein with antigenic similarity to CT would be useful in preparing lots for clinical use (6).

Conjugate vaccines have several advantages. (i) No serious adverse reactions are anticipated because the LPS levels are low (24). (ii) Conjugated saccharides can be expected to have greater immunogenicity and T-cell-dependent properties than do cellular vaccines (9, 14, 31, 49). The conjugate may represent a safer and more immunogenic (and thereby more effective) cellular vaccine. (iii) Conjugates may be administered concurrently with diphtheria-tetanus-pertussis vaccine and *H. influenzae* type b conjugates to infants (49). If shown to be protective, the *V. cholerae* LPS conjugate could be included into the routine immunization program for infants and children, the age group with the highest attack rate in areas endemic for cholera (38). (iv) The composition of our conjugate can be standardized so that the potency of new lots can be controlled by laboratory assays. (v) Lastly, should our conjugate prevent cholera, future lots may be evaluated in different populations by measuring the serum LPS or vibriocidal antibodies of recipients without the need to perform additional efficacy trials.

The epidemic of cholera in the Western Hemisphere, first recognized in Peru in 1991, illustrates the continuing menace of this disease. The strain of *V. cholerae* causing the epidemic is LPS type Inaba, CT-2, biotype El Tor (16, 29,

32). We plan to evaluate the protective efficacy of a conjugate vaccine composed of the detoxified LPS and CT of this strain.

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