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Comparative Immunogenicity of Conjugates Composed of *Escherichia coli* O111 O-Specific Polysaccharide, Prepared by Treatment with Acetic Acid or Hydrazine, Bound to Tetanus Toxoid by Two Synthetic Schemes

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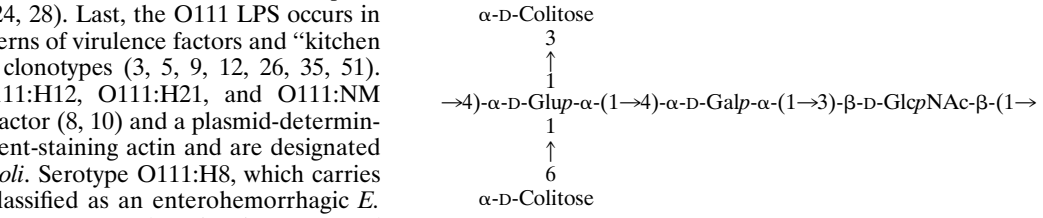
***Escherichia coli* O111, of various H types and virulence factors, causes enteritis throughout the world, especially in young children. This O type is found rarely in healthy individuals. Serum antibodies to the O-specific polysaccharide of O111 lipopolysaccharide (LPS) protect mice and dogs against infection with this *E. coli* serotype. The O111 O-specific polysaccharide is composed of a pentasaccharide repeat unit with two colitoses bound to the C-3 and C-6 of glucose in a trisaccharide backbone; this structure is identical to that of *Salmonella adelaide* (O35), another enteric pathogen. Nonpyrogenic O111 O-specific polysaccharide was prepared by treatment of its LPS with acetic acid (O-SP) or the organic base hydrazine (DeA-LPS). The O-SP had a reduced concentration of colitose. These products were derivatized with adipic acid dihydrazide (ADH) or thiolated with *N*-succinimidyl-3(2-pyridyldithio) propionate (SPDP). The four derivatives were covalently bound to tetanus toxoid (TT) by carbodiimide-mediated condensation or with SPDP to form conjugates. Immunization of BALB/c and general-purpose mice by a clinically acceptable route showed that DeA-LPS–TT_{ADH}, of the four conjugates, elicited the highest level of LPS antibodies. Possible reasons to explain this differential immunogenicity between the four conjugates are discussed.**

Strains of the O111:B4 serotype were among the first *Escherichia coli* strains identified as a cause of infantile diarrhea; they were designated as enteropathogenic *E. coli* (4, 25, 26, 31, 32, 39). For example, *E. coli* O111 recently accounted for 33% of diarrhea cases in children in Brazil (39), diarrhea in 600 children and 36 adults in an outbreak in Finland (48), and diarrhea in neonates in Kenya (43).

There is evidence that the O111 lipopolysaccharide (LPS) is both a virulence factor and a protective antigen. First, strains of *E. coli* O111 are almost always found in patients with enteritis; asymptomatic carriage of this O type is rare (35). Second, the O-specific polysaccharides of *E. coli* O111 and *Salmonella adelaide*, another enteric pathogen, are identical (34). Third, *E. coli* O111 strains are serum resistant, an in vitro property associated with virulence; this resistance is mediated by the O-specific polysaccharide and is related to its composition and M_r (17–19, 23, 24, 28). Last, the O111 LPS occurs in *E. coli* with different patterns of virulence factors and "kitchen enzymes" designated as clonotypes (3, 5, 9, 12, 26, 35, 51). Serotypes O111:H2, O111:H12, O111:H21, and O111:NM have localized adhesion factor (8, 10) and a plasmid-determining factor called fluorescent-staining actin and are designated as enteropathogenic *E. coli*. Serotype O111:H8, which carries the Shiga-like toxin, is classified as an enterohemorrhagic *E. coli* (3). Another serotype, O111:H19, found to be a cause of watery diarrhea in infants and associated with heat-labile (cholera-like) toxin, is designated as an enterotoxigenic *E. coli* (3). Despite the variation in their H types, that the O-specific polysaccharide of O111 is a protective antigen is shown by the fact that polyclonal and monoclonal antibodies induce bactericidal and opsonophagocytic activities against *E. coli* O111 and afford protection against experimental infections of this pathogenic LPS type in mice and dogs (8, 22, 33).

Serum immunoglobulin G (IgG) anti-O-specific polysaccharide provides protection in animals and humans against other gram-negative enteric pathogens (41, 46). For this reason, we devised methods to prepare clinically acceptable vaccines designed to elicit serum antibodies against the O-specific polysaccharide of *E. coli* O111, especially of the IgG class, for active immunization of individuals at risk, such as premature infants, and for passive immunization of patients with systemic infection with this pathogen.

We used the LPS of *E. coli* serotype O111:K58(B4):NM. The O111 O-specific polysaccharide is composed of a repeat unit of four neutral sugars (13, 14):



Colitose is 3,6-dideoxy-L-galactose. In addition to its LPS, *E. coli* O111:B4 produces a capsule-like polymer identical to the O-specific polysaccharide described above (17–19, 23, 24, 30, 35).

LPS is not a suitable vaccine because of its toxicity, and its O-specific polysaccharide is not immunogenic, probably because of its comparatively low M_r (52). The usual method for preparing O-specific polysaccharides is treatment with acetic acid to remove the lipid A by hydrolysis of its 3-deoxy-D-manno-2-octulosonic acid (KDO)-glucosamine linkage (50).

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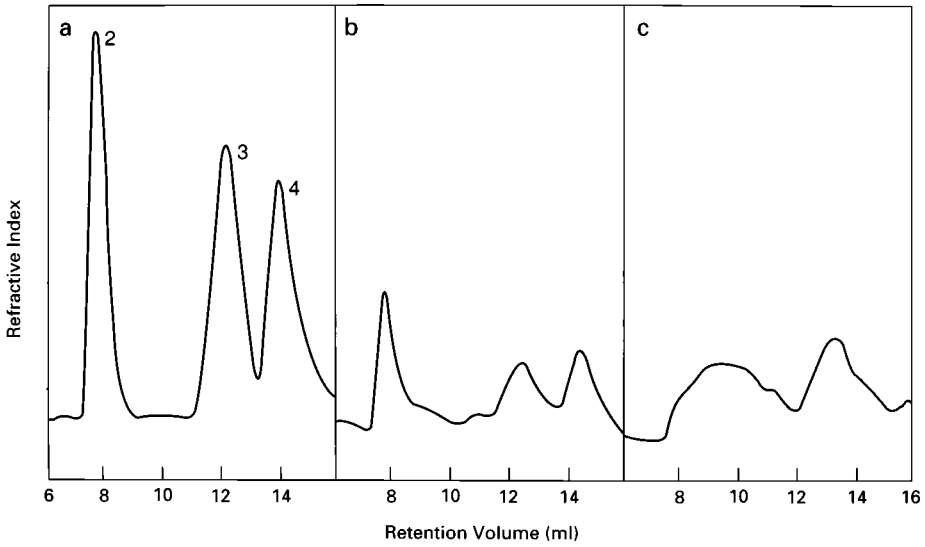

FIG. 1. HPLC profiles of *E. coli* O111:B4 LPS (100 μg) (a), DeA-LPS (25 μg) (b), and O-SP (25 μg) (c) through a Superose 12 column (10 by 300 mm) equilibrated in 0.2 M NaCl–1 mM EDTA–10 mM Tris–0.25% deoxycholic acid (pH 8.0).

We found that this procedure also removes some of the colitose from the O-specific polysaccharide. We prepared conjugates with O-specific polysaccharides from O111 treated with acetic acid (O-SP) (6, 44, 45) and with the organic base hydrazine (DeA-LPS) (20). O-SP and DeA-LPS were conjugated to carrier proteins by two schemes, one by multiple-point attachment by use of adipic acid dihydrazide (ADH) as a linker (6, 7, 38, 46) and the other by single-point attachment to aminoethanol of the core region by use of *N*-succinimidyl-3(2-pyridyldithio) propionate (SPDP) as a linker (15, 16, 20, 44, 45). The levels of immunogenicity in mice of these *E. coli* O111 conjugates were compared.

MATERIALS AND METHODS

Bacteria. *E. coli* O111:B4 was obtained from the American Type Culture Collection, Rockville, Md.

Antigens. Tetanus toxoid (TT), from Staten Seruminstitut, Copenhagen, Denmark, was fractionated through a column of S-300 Sephacryl (2.5 by 90 cm) equilibrated in 0.2 M NaCl (7). *E. coli* O111 LPS was from List Biological Laboratories, Campbell, Calif.

Reagents. The following reagents were used: sterile pyrogen-free water and pyrogen-free saline from Baxter Health-Care Corporation, Deerfield, Ill.; anhydrous hydrazine, ADH, dithiothreitol, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC), EDTA, KDO, and dextrans from Sigma Chemical Co., St. Louis, Mo.; sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and deoxycholic acid from Calbiochem, La Jolla, Calif.; SPDP and bicinchoninic acid protein assay reagent from Pierce Chemical Co., Rockford, Ill.; cyanogen bromide from Eastman Chemical, Rochester, N.Y.; G-25 Sephadex, Sephacryl S-300, and a high-performance liquid chromatography (HPLC) Superose 12 column (10 by 300 mm) from Pharmacia-LKB, Piscataway, N.J.; goat anti-mouse IgG and IgM alkaline phosphatase conjugates from Kirkegaard & Perry, Gaithersburg, Md.;*p*-nitrophenyl phosphate from Fluka, Ronkonkoma, N.J.; *E. coli* O111:B4 LPS from List Biological; *Limulus* amebocyte lysate from Associates of Cape Cod, Inc., Woods Hole, Mass.

Analyses. The K_d of TT was determined by gel filtration through a column of S-300 Sephacryl (2.5 by 90 cm) equilibrated in 0.2 M NaCl and, for the saccharides, through a column of Superose 12 (10 by 300 mm) equilibrated in a mixture of 0.2 M NaCl, 0.001 M EDTA, 0.01 M Tris, and 0.25% deoxycholic acid at pH 8.0. The contents of the two dideoxy sugars, KDO and colitose, were measured by the periodate-thiobarbituric acid assay with KDO as a standard (49). ADH was measured by the TNBS reaction with ADH as a standard (7). Protein was measured by the bicinchoninic acid reagent with bovine serum albumin (BSA) as a standard, and polysaccharide was measured by the anthrone reaction with O-SP as a standard (27). Silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with a 14% gel (Novex, San Diego, Calif.), was performed as described previously (47). The contents of the protein and nucleic acids of the saccharides were assayed by measuring their A_{280} and A_{255}, respectively (52).

NMR. O-SP and DeA-LPS were analyzed by ^{13}C nuclear magnetic resonance (NMR). The polysaccharides (ca. 15 mg) were dissolved in 0.5 ml of pyrogen-free water, and spectra were recorded on a General Electric GN300 spectrometer. Approximately 42,000 free-induction decay signals were averaged for each spectrum. Spectral acquisition parameters include 8,192 datum points, a 3-s delay between the end of the acquisition of one flame ionization detection signal and the start of the next, and a 90° (15-μs) pulse. Prior to Fourier transformation, the flame ionization detection signals were multiplied exponentially (3-Hz line broadening) and zero-filled to 32,000 datum points.

Detoxification of LPS. LPS was detoxified by two methods.

(i) Acetic acid treatment. LPS was suspended in 1% acetic acid and heated at 100°C for 90 min (6, 50). The treated LPS was ultracentrifuged at 60,000 × *g* for 5 h at 10°C to remove the remaining lipid. The excess reagents in the supernatant were removed by passing through a G-50 column equilibrated in 0.15 M NaCl. The void volume fractions, shown to contain polysaccharide, were dialyzed exhaustively against pyrogen-free water, sterile filtered, and freeze-dried. The acid-treated LPS was designated O-SP.

(ii) Hydrazine treatment. LPS was dried over P_2O_5 for 2 to 3 days, suspended in anhydrous hydrazine (10 mg/ml), and heated at 37°C for 2 h (20). The reaction mixture was precipitated and washed with 80% cold acetone and dissolved in 0.15 M NaCl at pH 7.0. The residual LPS and reagents were removed as described for O-SP (vide supra). The hydrazine-treated LPS was designated DeA-LPS.

Conjugation to TT. Conjugates were prepared by two methods.

(i) Method 1. The first scheme utilized the heterobifunctional reagent SPDP to thiolate the polysaccharide and the protein (44). DeA-LPS or O-SP (5 mg/ml) or protein (3 mg/ml) was dissolved in HEPES buffer–0.01 M EDTA (pH 7.5), and SPDP (0.02 M in 100% ethylalcohol) was added (weight ratio for SPDP/polysaccharide and SPDP/protein, 0.2) and passed through a gel filtration column. The extent of derivatization of the polysaccharide and protein with SPDP was determined spectrophotometrically (15, 44). The *N*-pyridyl disulfide bond of SPDP-polysaccharide was cleaved by 0.04 M dithiothreitol and mixed with an equal weight of protein-SPDP, and the reaction mixture was passed through a gel filtration column. Conjugates synthesized by this method were designated DeA-LPS–TT_{SPDP} and O-SP–TT_{SPDP}.

(ii) Method 2. O-SP and DeA-LPS were derivatized with ADH as described for *Haemophilus influenzae* type b polysaccharide (6, 7). O-SP or DeA-LPS, at 10 mg/ml of pyrogen-free saline, was activated with cyanogen bromide at pH 10.5 and derivatized with ADH in 0.5 M $NaHCO_3$ at pH 8.5. Excessive reagents were removed by gel filtration. Polysaccharide-ADH derivatives were dissolved to a concentration of 10 mg/ml. An equal volume of protein (~10 mg/ml) was added, and the pH was adjusted to 5.5. EDAC was added to a final concentration of 0.05 M, and the pH was maintained at 5.5 to 6.0. Gel filtration fractions containing both saccharide and protein were pooled. Conjugates synthesized by this method were designated O-SP–TT_{ADH} and DeA-LPS–TT_{ADH}.

Bioassays. The toxicity of LPS was assayed by the *Limulus* amebocyte lysate test and expressed in endotoxin units related to the U.S. standard (21). Pyrogenicity, assayed in rabbits, was kindly determined by Donald Hochstein, Center for Biologics Evaluation and Research, Bethesda, Md.

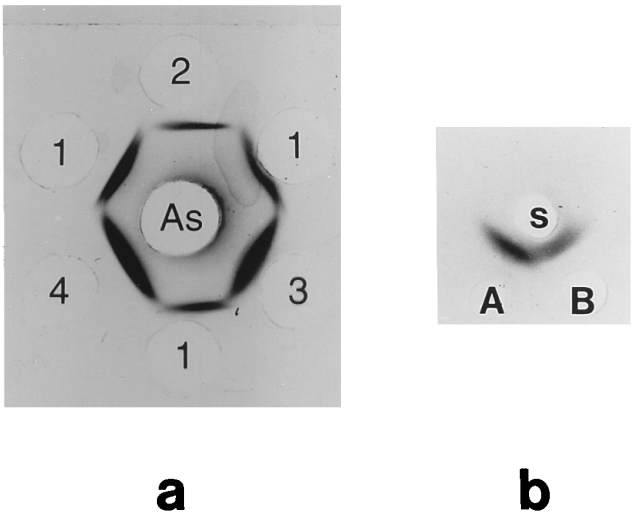

FIG. 2. Double immunodiffusion. Each well contains 10 μl. (a) Wells: center, hyperimmune murine O111 serum; 1, *E. coli* O111 LPS (1 mg/ml); 2, 3, and 4, fractions from HPLC column in Fig. 1. (b) Wells: center, hyperimmune murine O111 serum; A, DeA-LPS (1 mg/ml); B, O-SP (1 mg/ml).

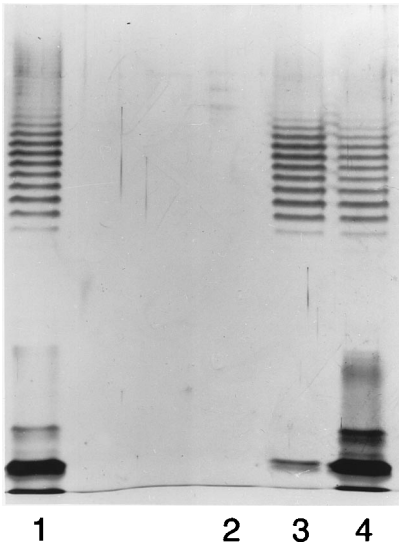

FIG. 3. Silver-stained 14% SDS gel of *E. coli* O111 LPS and fractions 2, 3, and 4 from gel filtration on a Superose 12 column (10 by 300 mm; see Results). Lanes: 1, *E. coli* O111 LPS (10 μg); 2 to 4, fractions 2 to 4 from Superose 12 column, respectively.

Serology. Double immunodiffusion was performed in 1% agarose in phosphate-buffered saline (PBS). LPS and protein antibody levels were determined by an enzyme-linked immunosorbent assay (ELISA). The plates were coated with LPS or protein at 1 μg per well in PBS. Serum was added in serial twofold dilutions, and the plates were incubated at 37°C for 4 h. Antibody levels were expressed in ELISA units, with the hyperimmune serum assigned a value of 100 U as a reference. The levels are expressed as the geometric mean and the 25th and 75th centiles.

Immunization. Hyperimmune sera were prepared by repeated injections of female, adult BALB/c mice with heat-killed bacteria, as described previously (6). For evaluation of immunogenicity, 6-week-old female BALB/c or general-purpose mice from the National Institutes of Health colony were injected subcutaneously with 2.5 μg of O-SP or DeA-LPS alone or as a conjugate in saline. Mice were reinjected twice at 10- to 14-day intervals and bled 7 days after each injection.

Statistical analyses. Antibody levels below the sensitivity of the ELISA were assigned half of that value. Comparison of geometric means were performed by an unpaired *t* test. The Statistical Analysis System was used for all data analysis.

RESULTS

Characterization of LPS, O-SP, and DeA-LPS. O111 LPS contained <1% nucleic acids and protein. HPLC showed three peaks: a high-molecular-size peak (K_d, <0.1) and two minor peaks of lower molecular size (K_d, 0.4 and 0.6) (Fig. 1a). All three peaks precipitated with hyperimmune mouse serum with an identity reaction (Fig. 2a). Silver-stained SDS-PAGE patterns of these peaks (Fig. 3) showed that only the lower-molecular-size fractions exhibited a ladder pattern characteristic of LPS.

The acid- and base-treated saccharides had reduced levels of LPS (<2 endotoxin units per μg) and were not pyrogenic in rabbit thermal tests. The molecular sizes and antigenicity of O-SP and DeA-LPS were analyzed by HPLC and immunodiffusion. DeA-LPS had three peaks with the same K_ds as those of the LPS (Fig. 1b). O-SP, in contrast, had two peaks with K_ds of 0.2 and 0.5 (Fig. 1c). By double immunodiffusion, spurs extended from the LPS over the DeA-LPS and O-SP when reacted with hyperimmune O111 serum (data not shown). DeA-LPS and O-SP formed a line of identity with each other (Fig. 2b).

Physicochemical analysis of LPS, O-SP, and DeA-LPS. Figure 4 shows the ^{13}C NMR spectra of O-SP and DeA-LPS. DeA-LPS and O-SP exhibited qualitatively similar spectra. There was a lesser amount of colitose in O-SP than in DeA-LPS, as shown by the signals at 18 ppm. DeA-LPS also exhibited fewer resonances (data not shown) of lower intensities in the spectral region (30 to 40 ppm) anticipated for residual amide-linked lipid than LPS did.

The dideoxy sugar content of DeA-LPS and LPS was 21%, and that of O-SP was 14% (Table 1). The reduction in dideoxy sugar content of O-SP could come from the loss of either KDO and/or colitose during acid hydrolysis since the colorimetric reaction (50) does not distinguish between these two saccharides. The phosphate concentrations were 8 μg/mg of polysaccharide for LPS and DeA-LPS and 3 μg/mg for O-SP.

Characterization of the conjugates. Table 2 lists the compositions of the four conjugates synthesized with DeA-LPS and O-SP. The derivatization with ADH was 2.17% for DeA-LPS and 1.70% for O-SP. The derivatization with SPDP was 0.27% for DeA-LPS and 0.60% for O-SP. The saccharide-to-protein ratios of the four conjugates were similar and ranged from 0.7 to 1.04.

The four conjugates reacted with the hyperimmune O111 and TT sera in double immunodiffusion with an identical line of precipitation (data not shown).

Serum LPS antibodies. Neither O-SP nor DeA-LPS alone elicited detectable antibodies in BALB/c or general-purpose mice (data not shown).

(i) BALB/c mice (Table 3). After one injection, only DeA-LPS–TT_{ADH} induced an antibody rise. After two injections, DeA-LPS–TT_{ADH} elicited booster responses for both IgG and IgM ($P < 0.01$). DeA-LPS–TT_{SPDP} and O-SP_{SPDP} did not elicit O111 antibodies, with the exception of a low level of IgM in the O-SP–TT_{SPDP} group after the third injection. After the third injection, antibody levels elicited by DeA-LPS–TT_{ADH} were higher than those elicited by the other three conjugates (for IgG, 24.6 versus 1.04, 0.17, and 0.07 [$P = 0.0001$]; for IgM, 8.31 versus 0.83, 0.24, and 0.80 [$P = 0.0001$]).

(ii) General-purpose mice (Table 4). The conjugates elicited similar responses in the general-purpose mice as those in the BALB/c mice. DeA-LPS–TT_{ADH} induced higher levels of IgG antibodies than other conjugates after each of the three injec-

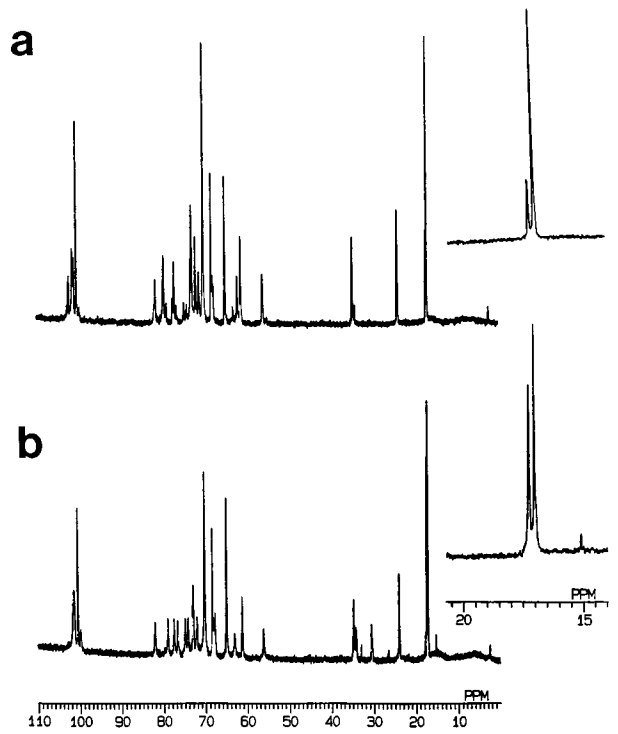

FIG. 4. ^{13}C NMR spectroscopy of O-SP (a) and DeA-LPS (b). The polysaccharides (ca. 15 mg) were dissolved in 0.5 ml of D_2O, and their spectra were recorded with a General Electric GN300 spectrometer. Approximately 42,000 free induction decay signals were averaged for each spectrum. Spectral acquisition parameters included 8,192 datum points, 3-s delay between the end of the acquisition of one flame ionization detection signal and the start of the next, and a 90° (15-μs) pulse. Prior to Fourier transformation, the flame ionization detection signals were multiplied exponentially (3-Hz line broadening) and zero-filled to 32,000 datum points. The inserts show peaks corresponding to those of colitose.

tions (post-third injection, 6.80 versus 0.25, 0.82, and 0.17 [$P < 0.001$]). In the general-purpose mice, DeA-LPS–TT_{ADH} elicited a rise of IgG O111 antibodies from 2.26 to 6.80 after the third injection, but this is not statistically significant ($P = 0.07$). Similarly, DeA-LPS–TT_{ADH} elicited higher IgM levels after each injection than the other three conjugates, but these differences were not statistically significant.

(iii) TT antibodies (Table 5). TT antibodies were not detected in any of the mice after one injection of the conjugates (data not shown). After two injections, all groups reacted with a rise in TT antibodies ($P < 0.004$). DeA-LPS–TT_{ADH} induced the highest level of TT antibodies in BALB/c mice compared with those of the other conjugates after three injections (6.64 versus 0.69, 2.01, and 1.13 [$P = 0.0001$]).

TABLE 1. Composition of *E. coli* O111 LPS and O-SP and DeA-LPS derivatives[a]

Polysaccharide	Amt (μg) of component/mg of total CHO[b]	
	Dideoxy sugar	Phosphate
LPS	212	8.0
DeA-LPS	211	8.5
O-SP	140	3.0

[a] The three saccharides contained less than 1% nucleic acid and protein (48).
[b] Deoxy sugars were measured by the periodate-thiobarbituric acid reaction with KDO as a standard (49). CHO, carbohydrate.

TABLE 2. Characterization of O-SP–TT_{SPDP}, O-PS–TT_{ADH}, DeA-LPS–TT_{ADH}, and DeA-LPS-TT_{SPDP}

Conjugate	Linker (%)	SPDP/TT (mol/mol)	PS/TT (wt/wt)
DeA-LPS-TT_{ADH}	2.17	NA[a]	0.70
O-SP-TT_{ADH}	1.70	NA	1.04
DeA-LPS-TT_{SPDP}	0.27	37	0.75
O-SP-TT_{SPDP}	0.6	37	0.90

[a] NA, not applicable.

DISCUSSION

E. coli strains of the O111 LPS type have combinations of virulence factors and cause clinically distinct diseases (3, 5, 9, 12, 29, 35, 39, 43, 51). Accordingly, *E. coli* O111 strains may be classified as enteropathogenic, enteroinvasive, enterotoxigenic, and enterohemorrhagic. It is likely that the O-specific polysaccharide serves as a protective antigen for all of these clones of pathogenic *E. coli* O111 as well as for *S. adelaide* (34). We proposed that serum IgG antibodies to the surface polysaccharides of enteric bacterial pathogens will confer protective immunity by killing the inoculum of these bacteria as the bacteria enter the jejunum (20, 40, 41). To provide clinically acceptable vaccines for either active or passive immunization of enteric infections caused by *E. coli* O111, we studied methods to prepare protein conjugates of its O-specific polysaccharide.

The O-specific polysaccharide from *E. coli* O111, prepared by treatment with either acetic acid (O-SP) or hydrazine (DeA-LPS), was derivatized with ADH or SPDP and bound to TT by two schemes (6, 7, 15, 16, 20, 38, 44–46). DeA-LPS–TT_{ADH} elicited statistically significant higher levels of O111 LPS antibodies than the other three conjugates. Several factors could explain this difference in immunogenicity. First, the amount of colitose was reduced in O-SP as measured by the thiobarbituric acid assay (49) and ^{13}C NMR. This reduction in the colitose, we think, is the most important factor in the lesser immunogenicity of the O-SP-based conjugates. Second, DeA-LPS has a higher M_r than that of O-SP (20, 38). Although the size of the saccharide moiety of conjugates has been shown to be related to its immunogenicity (1, 36, 52), Polotsky et al. showed that conjugates prepared with O-SP of *Shigella flexneri* 2a were more immunogenic than those prepared with DeA-LPS, even though the latter saccharides had higher M_rs (38). Third, in

TABLE 3. Serum IgG and IgM antibodies to *E. coli* O111 LPS elicited in BALB/c mice immunized with various conjugates[a]

Vaccine	Injection		ELISA units (25th–75th centiles)	
	No.	*n*	IgG	IgM
DeA-LPS–TT_{ADH}	1	3	0.53 (0.05–5.23)	1.10 (0.65–1.63)
	2	3	8.90 (6.32–10.8)	2.19 (0.67–4.33)
	3	11	24.62 (14.1–34.1)	8.31 (5.58–12.14)
O-SP–TT_{ADH}	1	3	0.05 (0.05–0.05)	0.50 (0.47–0.53)
	2	3	0.12 (0.05–0.25)	0.49 (0.40–0.58)
	3	11	1.04 (0.59–1.93)	0.83 (0.52–1.17)
DeA-LPS–TT_{SPDP}	1	3	0.05 (0.05–0.05)	0.04 (0.04–0.05)
	2	3	0.10 (0.05–0.46)	0.09 (0.08–0.10)
	3	11	0.12 (0.05–0.24)	0.24 (0.14–0.32)
O-SP–TT_{SPDP}	1	3	0.05 (0.05–0.05)	0.15 (0.13–0.18)
	2	3	0.05 (0.05–0.05)	0.54 (0.19–1.62)
	3	11	0.07 (0.05–0.05)	0.80 (0.45–1.18)

[a] The IgG and IgM anti-LPS antibodies elicited by DeA-LPS-TT_{ADH} after each injection were higher than those elicited by the other three conjugates ($P = <0.01$).

TABLE 4. Serum IgG and IgM antibodies to the LPS of *E. coli* O111 elicited in general-purpose mice with various conjugates

Vaccine	Injection		Geometric mean ELISA units (25th–75th centiles)	
	No.	*n*	IgG[a]	IgM[b]
DeA-LPS–TT_{ADH}	1	3	0.05 (0.05–0.05)	0.09 (0.05–0.29)
	2	3	2.26 (1.85–3.09)	0.58 (0.46–0.74)
	3	11	6.80 (5.59–23.98)	0.87 (0.05–3.42)
O-SP–TT_{ADH}	1	3	0.05 (0.05–0.05)	0.05 (0.05–0.05)
	2	3	0.21 (0.05–3.53)	0.10 (0.05–0.45)
	3	11	0.25 (0.05–3.23)	0.08 (0.05–0.05)
DeA-LPS–TT_{SPDP}	1	3	0.05 (0.05–0.05)	0.05 (0.05–0.05)
	2	3	0.05 (0.05–0.05)	0.05 (0.05–0.05)
	3	10	0.82 (0.05–4.34)	0.07 (0.05–0.08)
O-SP–TT_{SPDP}	1	3	0.05 (0.05–0.05)	0.05 (0.05–0.05)
	2	3	0.05 (0.05–0.05)	0.28 (0.05–0.95)
	3	11	0.17 (0.05–0.83)	0.42 (0.28–0.98)

[a] For IgG, 6.80 versus 0.25, 0.82, and 0.17, $P = 0.001$.
[b] For IgM, 0.87 versus 0.08, 0.07, and 0.42, P is not significant.

contrast to O-SP, DeA-LPS retains N-linked acyl chains, the D-glucosamine disaccharide, and phosphoryl groups of the inner core. This residual lipid A may retain some adjuvant activity of the LPS. Fourth, ADH has been shown to be a more effective linker, possibly because of its greater chain length and more flexible hydrocarbon linkage compared with the disulfide bond in SPDP (15, 20, 38). Fifth, the extent of derivatization of polysaccharides is higher with ADH than with SPDP. This is probably because ADH binds vicinal hydroxyls, which are more numerous than amino groups, (amino groups are only on the aminoethanol of the core region). This difference in the derivatization of the two linkers affects the attachment of polysaccharides to TT in the two pairs of conjugates. With ADH as a linker, TT binds throughout the polysaccharide chain. With SPDP as a linker, the attachment of TT was only through the terminal amino groups at the nonreducing end of the polysaccharide. We conclude that the higher retention of colitose, higher M_r, multipoint attachment of DeA-LPS to TT, and the physicochemical properties of ADH all account for the superior immunogenicity of DeA-LPS–TT_{ADH} over that of the other three conjugates. It is not yet possible to predict the immunogenicity of the saccharide component by in vitro methods, so it will be necessary to compare the immunogenicity analyses of new conjugates.

There are two related surface polysaccharides of *E. coli* O111:B4, an LPS and a capsule-like structure composed of the same O-specific polysaccharide that does not contain the core or the lipid A regions (17–19, 24, 30, 35). We found this capsule-like O antigen in another pathogenic *E. coli* O157:H7 strain (27).

TABLE 5. Serum TT antibodies (G.M.) elicited in mice injected with conjugates of O-SP and DeA-LPS of *E. coli* O111 LPS[a]

Vaccine	Injection		ELISA units (25th–75th centiles) of TT antibodies	
	No.	*n*	BALB/c mice	GPM
DeA-LPS–TT_{ADH}	2	3	1.83 (1.59–2.18)	1.29 (0.65–1.98)
	3	11	6.64 (5.27–9.96)	4.02 (3.54–4.75)
O-SP–TT_{ADH}	2	3	0.52 (0.44–0.58)	1.29 (0.82–2.04)
	3	11	0.69 (0.54–0.82)	2.52 (1.92–3.48)
DeA-LPS–TT_{SPDP}	2	3	0.53 (0.47–0.63)	1.64 (0.94–3.47)
	3	10	2.01 (1.50–2.64)	2.76 (2.14–5.56)
O-SP–TT_{SPDP}	2	3	0.41 (0.19–0.76)	3.26 (1.68–4.86)
	3	11	1.13 (0.85–1.70)	4.20 (3.38–6.26)

[a] TT antibodies were not detected after the first injection in all groups. For BALB/c mice, 6.64 versus 0.69, 2.01, and 1.13, $P = 0.0001$; 2.01 versus 0.69, $P = 0.0001$. For general-purpose mice (GPM), 4.02 versus 2.52, $P = 0.01$; 4.20 versus 2.52, $P = 0.01$.

Leive and Limenez-Lucho showed that the O-specific moieties on LPSs from *E. coli* O111 and *Salmonella typhimurium* exert anticomplementary effects similar to those of the capsular polysaccharides of pathogenic bacteria (28). The resistance of the organism to the actions of the alternate complement pathway was mediated by their LPSs and was related to both the structure and length of the O-specific polysaccharide (17–19, 23, 24, 28). Serum antibodies against the O-specific side chain may render the bacterial surface more susceptible to complement attack of these pathogens and thereby confer protective immunity to *E. coli* O111, K1-bearing *E. coli*, and *S. typhimurium* (23, 37).

Serum antibody responses of healthy adult volunteers injected with heat-killed *E. coli* O111:B4 and other members of the family *Enterobacteriaceae* were mostly IgM, and reinjection did not elicit a booster response (2, 11, 42). Conjugates of capsular polysaccharides and of O-specific polysaccharides, in contrast, elicit mostly IgG antibodies, which persist longer than IgM antibodies (1, 6, 7, 16, 40, 41). Serum IgG can also penetrate the intestinal membrane and protect against the disease by inactivating the inoculum. We did not analyze the distribution of IgG subclasses of anti-LPS elicited by conjugates. This IgG subclass composition is potentially important, as shown in protection studies where IgG2a is the most efficient antibody against challenge of mice with *E. coli* O111 strains (33). We plan to study this problem in our clinical studies of DeA-LPS–TT_{ADH}. In humans, conjugates elicit booster responses in infants and young children only (10, 46). On the basis of these experimental data, we predict that DeA-LPS–TT_{ADH} will elicit high levels of IgG LPS antibodies in adults and booster responses in infants. Clinical evaluation of the safety and immunogenicity of *E. coli* O111 conjugates is planned.

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