Syntheses and Immunologic Properties of *Escherichia coli* O157 O-Specific Polysaccharide and Shiga Toxin 1 B Subunit Conjugates in Mice

EDWARD KONADU,¹ ARTHUR DONOHUE-ROLFE,² STEPHEN B. CALDERWOOD,³
VINCE POZSGAY,¹ JOSEPH SHILOACH,⁴ JOHN B. ROBBINS,¹ AND SHOUSUN C. SZU^{1*}

National Institute of Child Health and Human Development¹ and National Institute of Diabetes and Digestive and Kidney Diseases,⁴ National Institutes of Health, Bethesda, Maryland 20892-2720; Tufts University School of Veterinary Medicine, Medford, Massachusetts 01536²; and Massachusetts General Hospital, Boston, Massachusetts 02114³

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***Escherichia coli* O157 is the major cause of diarrhea-associated hemolytic uremic syndrome (HUS). Strains causing HUS contain either Shiga toxin 1 (Stx1) or Stx2, or both. In adult volunteers, conjugate vaccines of detoxified lipopolysaccharide (LPS) elicited bactericidal antibodies to *E. coli* O157. Here, the detoxified LPS was conjugated with improved schemes to the nontoxic B subunit of Stx1. Mice injected with these bivalent conjugates elicited both bactericidal antibodies to *E. coli* O157 and neutralization antibodies to Stx1.**

Escherichia coli O157, an emerging pathogen, causes severe hemorrhagic colitis and the extraintestinal complication of hemolytic uremic syndrome (HUS) in 5 to 10% of patients (11, 16, 26). Pathogens that cause hemorrhagic colitis and HUS, including *E. coli* O157, O111, and O26 and *Shigella dysenteriae* type 1, produce Shiga toxin 1 or 2 (Stx1 or Stx2, respectively), or both (6, 7, 10, 19, 25, 28). The source of *E. coli* O157 is mainly contamination of food or of drinking water by bovine feces (5, 26). Treatment of infection with *E. coli* O157 has been difficult because antibiotics do not change the course of the enteritis of *E. coli* O157 or *S. dysenteriae* type 1 and may increase the incidence of HUS caused by these two pathogens (5, 22). This untoward effect has been proposed to be mediated by antibiotic-induced bacteriolysis and release of intracellular Shiga toxins.

O-specific polysaccharide (O-SP) conjugates for *E. coli* O157 infections are designed to elicit serum immunoglobulin G (IgG) anti-lipopolysaccharide (anti-LPS) that will inactivate the inoculum on the intestinal epithelium (12, 14, 24). A phase 1 clinical trial of *E. coli* O157 O-SP bound to *Pseudomonas aeruginosa* recombinant exoprotein A (*rEPA*) showed these conjugates to be safe and to elicit statistically significant increases in levels of IgG anti-LPS with bactericidal activity in all of 87 adult recipients: 80% responded with ≥ 4 -fold increases in anti-LPS levels within 1 week (14).

One concern related to the LPS-based vaccine is that induction of IgG anti-LPS with bactericidal activity could increase the incidence of HUS through toxin released upon lysis of the pathogen. Accordingly, in this study we synthesized conjugates of *E. coli* O157 O-SP bound to the nontoxic B subunit of Stx1 (Stx1B) that could induce both serum IgG anti-LPS with bactericidal activity and neutralizing antibodies to Stx1 (1, 2, 29). We modified the conjugation schemes from our earlier studies for the following reasons. (i) In the new bivalent conjugate, it was just as important to retain and to improve the immunogenicity of Stx1B as it was to retain and improve that of O-SP. (ii) Stx1B is substantially smaller than the carrier protein *rEPA*

used in a previous clinical study and thus contains fewer reaction sites for conjugation than *rEPA*. Furthermore, based on our past experiences, the molecular weight of the carrier protein influences the immunogenicity and the final yield of the conjugate. We have therefore chosen conjugation schemes that would minimize modifications to the carrier protein and would result in higher yield.

E. coli O157 O-SP was prepared by treatment of LPS with acetic acid (12, 14, 20). Stx1B was synthesized by *Vibrio cholerae* 0395-N1 (pSBC32 containing the Stx1B gene) and purified by affinity chromatography (1, 3, 17, 21). Sodium dodecyl sulfate–7% polyacrylamide gel electrophoresis of Stx1B showed one major band at 9 kDa and a faint band with a slightly higher molecular mass which does not correspond to the A subunit of Stx1 (data not shown).

For conjugation, O157 O-SP was bound to the Stx1B directly by treatment with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) or by carbodiimide-mediated condensation, with adipic acid dihydrazide (ADH) as the linker (13, 15). For direct conjugation, CDAP (100 mg/ml in acetonitrile) was added to the O-SP in saline (5 mg/ml) at 0.3/1 (wt/wt) at room temperature, pH 5.8 to 6.0. Sixty microliters of 0.2 M triethylamine was added to bring the pH to 7.0, a level which was maintained for 2 min. An equal weight of Stx1B was added to the CDAP-treated O-SP, and the pH was maintained at 8.0 to 8.5 for 2 h. The reaction mixture was passed through a 1.5- by 90-cm Sepharose 6B column in 0.2 M NaCl, and the void volume fractions were collected together and designated OSP-Stx1B.

Conjugate with ADH as the linker was prepared by modifying the scheme for O-SP-*rEPA* conjugate described previously (12, 14). Briefly, after the addition of triethylamine in the above procedure, an equal volume of 0.8 M ADH in 0.5 M NaHCO₃ was added and the pH was maintained at 8.0 to 8.5 for 2 h. The reaction mixture was dialyzed against saline overnight at 4°C and passed through a 2.5- by 31-cm P10 column in water. The void volume fractions were pooled, freeze-dried, and together designated OSP-AH. The level of derivatization of O-SP with ADH was similar (3.1 [wt/wt]) to that of our previous *E. coli* O157 preparations (14). OSP-AH (10 mg), dissolved in 2 ml of saline, was added to an equal weight of Stx1B, and the pH was brought to 5.1. The reaction mixture was put on ice, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiim-

* Corresponding author. Mailing address: National Institute of Child Health and Human Development, Room 424, Building 6, National Institutes of Health, Bethesda, MD 20892-2720. Phone: (301) 496-1185. Fax: (301) 402-9108. E-mail: scszu@helix.nih.gov.

TABLE 1. GM levels of IgG and IgM antibodies to *E. coli* O157:H7 LPS elicited in sera of mice injected with conjugates of O157:H7 O-SP bound to Stx1B

Substance or immunogen injected	Anti-LPS level (EU) ^a	
	IgG	IgM
Saline	<1	<1
OSP-AH-Stx1B	166 (34–325)	25 (12–75)
OSP-Stx1B	63 (22–211)	14 (10–55)

^a GM of levels measured in sera from 10 mice. Values in parentheses are 25th to 75th percentile ranges. IgG and IgM of pooled sera from hyperimmune *E. coli* O157:H7 mice were used as standards and assigned a value of 1,000 EU each.

ide (EDC) was added to a final concentration of 0.05 M, and the pH was maintained at 5.1 to 5.5 for 2 h. The reaction mixture was passed through a 1.5- by 90-cm Sepharose 6B column in 0.2 M NaCl, and the void volume fractions were collected and together designated OSP-AH-Stx1B. The saccharide/protein ratios were 0.5 (wt/wt) and 0.51 (wt/wt) for OSP-Stx1B and OSP-AH-Stx1B, respectively. The yields, based on saccharide in the conjugates, were 2.3% for OSP-Stx1B and 3.4% for OSP-AH-Stx1B. Both OSP-AH-Stx1B and OSP-Stx1B formed lines of identity when reacting with rabbit anti-Stx1 and mouse hyperimmune serum against *E. coli* O157 (data not shown).

Female general purpose mice ($n = 10$ /group) were injected subcutaneously with saline or one of the conjugates containing 2.5 μ g of saccharide on days 0, 14, and 28. The mice were exsanguinated 7 days after each injection. Pooled sera from hyperimmunized mice were used as a reference, and 1,000 enzyme-linked immunosorbent assay units (EU) were assigned to each IgG and IgM. Neutralization of Stx1 and Stx2 was measured with HeLa (CCL-2) cell monolayers in 96-well, flat-bottom microtiter plates (9). Each well was seeded with 1×10^4 to 6×10^4 cells in a 0.1-ml volume. Monolayers were established by overnight incubation in 5% CO₂. Toxin neutralization was determined by incubating dilutions of mouse serum with Stx1 or Stx2, each at a final concentration of 100 pg/ml. The serum-and-toxin mixture was incubated at room temperature for 30 min and a 0.1-ml volume was added to each well. Following incubation overnight, the surviving cells were determined spectrophotometrically by crystal violet staining (9). Toxin neutralization was determined from the dose-response curve of either Stx1 or Stx2 on each 96-well plate, and titer was expressed as the highest serum dilution to yield 50% neutralization. Representative serum samples (two from each group) were assayed for their complement-assisted bactericidal activity as described previously (12, 14).

After three injections, both conjugates elicited statistically significant increases in levels of IgG and IgM anti-LPS (Table 1). The geometric mean (GM) anti-LPS levels elicited by OSP-Stx1B were 63 EU for IgG and 14 EU for IgM, and those elicited by OSP-AH-Stx1B were 166 EU for IgG and 25 EU for IgM: the differences between two conjugates were not statistically significant. There was no detectable complement-dependent bactericidal activity in sera from mice injected with saline. Sera from mice injected with OSP-Stx1B or OSP-AH-Stx1B elicited bactericidal antibodies to *E. coli* O157 (Table 2). This activity was removed by absorption with *E. coli* O157 LPS.

Sera from mice injected with saline showed no neutralization of Stx1 or Stx2. All sera from mice injected with conjugates showed neutralization of Stx1 (Table 3). At a 1/1,000 dilution, the GM level of neutralization was 90% for OSP-AH-Stx1B and 98% for OSP-Stx1B (not shown). Mice injected with

TABLE 2. Complement-dependent bactericidal activity against *E. coli* O157:H7 elicited in serum of mice injected with O157 O-SP conjugated to Stx1B

Substance or immunogen injected and serum sample	Activity of anti-LPS (EU) ^a		Bactericidal titer ^b
	IgG	IgM	
Saline	<1	<1	<10
OSP-AH-Stx1B			
1	285	14	50
2	155	15	50
OSP-Stx1B			
3	265	12	200
4	241	28	200

^a IgG or IgM anti-LPS levels of serum samples from four individual mice were assayed for bactericidal titer.

^b The reciprocal bactericidal titer is the highest serum dilution yielding 50% killing of *E. coli* O157:H7. Absorption with LPS removed the bactericidal activity from sera (titer, <10).

OSP-Stx1B had a significantly higher GM neutralization antibody titer than those injected with OSP-AH-Stx1B (14,000 versus 8,040; $P = 0.03$). None of the serum samples from mice injected with either conjugate showed neutralization of Stx2.

In summary, both conjugates composed of OSP and Stx1B elicited anti-LPS with bactericidal activity to *E. coli* O157 and neutralizing antibodies to Stx1. The immunogenicity of each component of the conjugate was related to configuration and

TABLE 3. Neutralization titers^a of Stx1 in sera from mice injected with *E. coli* O157 O-SP conjugated with Stx1B

Immunogen	Serum	Titer ^b
OSP-AH-Stx1B	2a	10,000
	2b	12,000
	2c	6,400
	2d	12,000
	2e	15,000
	2f	16,000
	2g	1,000
	2h	6,400
	2i	4,000
	2j	>20,000
	Geometric mean	8,040*
OSP-Stx1B	5a	15,000
	5b	18,000
	5c	>20,000
	5d	12,000
	5e	18,000
	5f	16,000
	5g	>20,000
	5h	16,000
	5i	16,000
	5j	4,000
	Geometric mean	14,400**

^a Neutralization of Stx1 or Stx2 was measured by using HeLa cell monolayers incubated with dilutions of 100 pg of toxin/ml of serum. Sera from mice immunized with saline or LPS alone showed titers of <10. None of the sera showed neutralization of Stx2 (titers were <10). The titers are the highest serum dilutions to yield 50% neutralization.

^b ** versus *, $P < 0.03$.

the chemical linkage between polysaccharide and protein. OSP-Stx1B, prepared by direct conjugation of the saccharide to the protein, elicited higher levels of neutralizing antibodies to Stx1 than OSP-AH-Stx1B that used ADH as a linker. One possible explanation is that in OSP-AH-Stx1B synthesis, the ADH-derivatized polysaccharide was bound to the carboxyl amino acids on Stx1B through EDC condensation. Carboxyl amino acids may be important for the immunogenicity of Stx1B. In addition, treatment with EDC condensation during synthesis of OSP-AH-Stx1B could have caused protein aggregation and altered its immunologic properties. OSP-Stx1B, on the other hand, was synthesized without utilizing EDC, and synthesis was mostly through the lysine groups when Stx1B was conjugated with the polysaccharide. Similar results have been found in conjugates of *Salmonella paratyphi* A O-SP and *Staphylococcus aureus* capsular polysaccharides (13). Direct conjugation of O-SP to the protein seems to be the best compromise for synthesizing these bifunctional conjugates.

As predicted, the B subunit elicited neutralizing antibodies to the homologous Stx1 but not neutralizing antibodies to Stx2 (25, 29). Our experience with conjugates of the capsular polysaccharide from *Salmonella typhi*, Vi, with cholera toxin and its B subunit is that the holotoxin is a more effective carrier than its B subunit for both saccharide and neutralizing antibodies (27). Accordingly, we plan to use nontoxic mutants of Shiga holotoxins 1 and 2 as carrier proteins for *E. coli* O157 O-SP conjugates (17, 18). Concurrent vaccination with these proteins plus our *E. coli* O157 O-SP-Shiga mutant toxin conjugates could enhance the immune responses of both the anti-LPS and the antitoxins (4, 27). These anti-toxins could also be used to neutralize other non-O157 Shiga toxin-producing organisms (6, 7, 10, 23, 28). *Shigella sonnei*-rEPA conjugates were effective in significantly reducing the number of shigellosis cases in adults within 7 to 14 days of vaccination (8). Similarly, in the clinical trial of *E. coli* O157, the conjugate vaccines elicited significant antibody level increases within 1 week (12). We propose that *E. coli* O157 conjugate vaccines may be useful in controlling outbreaks and epidemics. Such conjugate formulations could also be considered for making hyperimmune therapeutic sera.

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