Published in final edited form as:

Carbohydr Res. 2010 July 19; 345(11): 1600–1608. doi:10.1016/j.carres.2010.05.006.

Immunochemical studies of *Shigella flexneri* 2a and 6, and *Shigella dysenteriae* type 1 O-specific polysaccharide-core fragments and their protein conjugates as vaccine candidates

Joanna Kubler-Kielb^{1,*}, Evgeny Vinogradov², Christopher Mocca¹, Vince Pozsgay¹, Bruce Coxon¹, John B. Robbins¹, and Rachel Schneerson¹

¹The Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD, 20892, USA

²Institute for Biological Sciences, National Research Council, 100 Sussex Dr., Ottawa ON, K1A 0R6, Canada

Abstract

There is no licensed vaccine for the prevention of shigellosis. Our approach to the development of *Shigella* vaccine is based on inducing serum IgG antibodies to the O-specific polysaccharide (O-SP) domain of their lipopolysaccharides (LPS). We have shown that low molecular mass O-SP-core (O-SPC) fragments isolated from *Shigella sonnei* LPS conjugated to proteins induced significantly higher antibody levels in mice than the full length O-SP conjugates. This finding is now extended to the O-SPC of *S. flexneri* 2a and 6, and *S. dysenteriae* type 1. The structures of O-SPC, containing core plus 1–4 O-SP repeat units (RU), were analyzed by NMR and mass spectroscopy. The first RUs attached to the cores of *S. flexneri* 2a and 6 LPS were different from the following RUs in their O-acetylation and/or glucosylation. Conjugates of core plus more than 1 RUs were necessary to induce LPS antibodies in mice. The resulting antibody levels were comparable to those induced by the full length O-SP conjugates. In *S. dysenteriae* type 1, the first RU was identical to the following RUs, with the exception that the GlcNAc was bound to the core in the β -configuration, while in all other RUs the GlcNAc was present in the α -configuration. In spite of this difference, conjugates of *S. dysenteriae* type 1 core with 1, 2, or 3 RUs induced LPS antibodies in mice with levels statistically higher than those of the full size O-SP conjugates. O-SPC conjugates are easy to prepare, characterize, and standardize, and their clinical evaluation is planned.

Introduction

Shigella is a major cause of diarrhea and dysentery worldwide, affecting millions of individuals, especially young children, and causing over a million deaths annually [1–3]. The infectious dose of Shigellae is very low, and ingestion of just 10 organisms may cause severe diarrhea and dehydration. The most dangerous form of the disease, dysentery, is

*Corresponding author: Tel: (301)-496-6141, Fax: (301)-480-3442, kielbj@mail.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

We have shown that a low mass O-SP-core fragments isolated from *Shigella sonnei* LPS conjugated to proteins induced significantly higher antibody levels in mice than full length O-SP conjugates. This finding is now extended to the O-SPC of *S. flexneri* 2a and 6, and *S. dysenteriae* type 1.

characterized by bloody and mucous diarrhea, abdominal cramps, rectal pain, and fever; it is also a major cause of stunted growth [4].

Shigellae are divided into four groups: Group A - *Shigella dysenteriae*, Group B - *Shigella flexneri*, Group C - *Shigella boydii*, and Group D - *Shigella sonnei* [5]. The groups are further divided into serotypes based upon the structure of the O-SP of their LPS. The *Shigella* O-SP is an essential virulence factor and a protective antigen [6].

Despite the discovery of the Shiga bacillus (*S. dysenteriae* type 1) in Japan over a century ago, there is still no licensed vaccine for shigellosis [2]. *S. sonnei* is the most common type in industrialized countries, as is *S. flexneri* 2a in developing countries. The most severe disease is caused by *S. dysenteriae* type 1, which is endemic and epidemic in developing countries. Experimental vaccines composed of protein conjugates of the O-SPs of *S. dysenteriae* type 1, *S. sonnei*, and *S. flexneri* 2a were safe, and induced specific LPS antibodies in young adults. The *S. sonnei* conjugate was > 70 % protective in young adults and in 3–4 year olds [3,6]. More immunogenic vaccine candidates were prepared using synthetic *S. dysenteriae* type 1 saccharides containing 2, 3, or 4 O-SP RU and low molecular mass O-SP-core (O-SPC) fragments containing an average of 3–4 RUs of *S. sonnei* LPS bound to carrier proteins [7,8]. Now, we examine if the latter approach can be extended to other Shigellae: *S. flexneri* types 2a and 6, and *S. dysenteriae* type 1.

Material and Methods

Bacteria and isolation of LPS

S. flexneri 2a strain 2457T10 and *S. dysenteriae* type 1 strain 1617 were from Dr. Sam Formal (WRAIR), *S. flexneri* 6 was a clinical isolate from a child with shigellosis in Israel [3]. All strains were cultivated as described [9]. LPSs were extracted by the hot phenol method and purified as described [10]. The average yields of LPS for each strain were 1.3–1.5% of wet cell mass.

Isolation of oligosaccharides

LPS (200 mg) was treated with 1% acetic acid at 100°C for 1.5 h. Lipid A was removed by ultracentrifugation at 35,000 rpm for 5 h at 4°C, and the soluble product subjected to gel chromatography on a BioGel P-10 column (1×100 cm) in pyridine/acetic acid/water buffer (4/8/988 mL), monitored with a Knauer differential refractometer. Fractions containing core plus one RU (core-1RU) were further purified by Hi-Trap Q anion-exchange chromatography using a 0–1 M NaCl gradient in water. The ratios of oligosaccharides obtained after gel filtration were as follows: for *S. flexnerii* 6: full length O-SP comprised 60.8%; F2, 3.6%; F3, 6.3% and F4, 14%; for *S. flexnerii* 2a: full length O-SP comprised 57.8%; F2, 7.3 %; F3, 7.4% and F4, 9.0%; for *S. dysenteriae* type 1: full length O-SP comprised 66.1%; F2 5.8 %; F3, 11.7% and F4, 15.5%.

Analytical methods

Protein concentration was measured by the method of Lowry [11], sugar concentration by the anthrone assay [12]. SDS-PAGE used 14% gels according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Immunodiffusion was performed in 1% agarose in PBS.

Methylation and monosaccharide analysis

Methylation was performed using the DMSO-NaOH-MeI procedure [13]. Methylated and non-methylated oligosaccharides were hydrolyzed with 3M TFA at 120°C for 3h and converted into alditol acetates [13]. Samples were analyzed by GLC-MS using an Agilent

6850 chromatograph equipped with DB-17 (30m × 0.25mm) fused-silica column using a temperature gradient of 180°C → 240°C at 2°C/min or by a Varian Saturn 2000 GC-MS using the same column.

NMR Spectroscopy

¹H and ¹³C NMR spectra were recorded using a Varian Inova 600 MHz spectrometer for samples in D₂O at 25–35 °C with acetone as an internal reference (2.225 ppm for ¹H and 31.5 ppm for ¹³C) and standard pulse sequences for double quantum filtered COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 400 ms), gradient HSQC, and HMBC (100 ms long range transfer delay).

Mass spectrometry

Capillary electrophoresis - mass spectrometry (CE-MS) used a 4000 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, ON, Canada) with a Prince CE system (Prince Technologies, Amsterdam, The Netherlands) with a 90 cm length, bare fused-silica capillary, and 15 mM ammonium acetate in deionized water, pH 9.0, in the injection module. A sheath solution (isopropanol-methanol, 2:1 v/v) was delivered at a flow rate of 1.0 µL/min. A –5 kV electrospray ionization voltage was used for negative ion detection modes.

MALDI-TOF mass spectra of the derivatized proteins and of the conjugates were obtained with an OmniFlex MALDI-TOF instrument (Bruker Daltonics, Billerica, MA) operated in the linear mode. Samples for analysis were desalted, and a 1 µL aliquot, mixed with 20 µL of sinnapinic acid matrix made in 30% CH₃CN and 0.1% TFA. Of this mixture, 1 µL was applied to and dried on the sample stage, then placed in the mass spectrometer.

Preparation of conjugates

Conjugates were prepared similarly to those of *S. sonnei* O-SPC [8]. To 15 mg of BSA (Sigma, St. Louis, MO) in 2.2 mL Buffer A (PBS, 0.1% glycerol, 5 mM EDTA, pH 7.2), 4 mg of SBAP (Pierce, Rockford, IL) in 40 µL DMSO were added and reacted with mixing for 1.5 h, pH 7.2, at room temp. Next, the reaction mixture was applied to a Sephadex G-50 column (1 × 50 cm) in 0.2 M NaCl, and the void volume fractions (BSA-Br) concentrated using an Amicon Ultra-15 centrifuge filter device (Millipore, Billerica, MA) to 2.6 mL (13 mg recovered); 0.2 mL was removed for analysis. An average of 30 linkers per BSA molecule was introduced under these conditions, as assayed by MALDI-TOF. To 12 mg of BSA-Br in 2.4 mL Buffer A, 10 mg *O*-(3-thiopropyl)hydroxylamine, prepared in our laboratory [14], dissolved in 300 µL of 1M K₂HPO₄, was added and reacted with mixing for 3 h, pH 7.2, at room temp. The reaction mixture was then passed through the Sephadex G-50 column and the void volume fractions (BSA-ONH₂) concentrated to 2.6 mL as above, and 0.2 mL removed for analysis. BSA-ONH₂, 10 mg, was reacted with 25 mg O-SPC in 3 mL of Buffer A, overnight, pH 5.6, at room temp, with mixing. The solution was then passed through Sepharose G-75 (1 × 100 cm) in 0.2 M NaCl and the void volume fractions collected and analyzed for sugar, protein and molecular mass by MALDI-TOF and SDS-PAGE. The following conjugates obtained in this way were designated: **1:** BSA/*S. flexneri* 2a O-SPC-F2; **2:** BSA/*S. flexneri* 2a O-SPC-F3, **3:** BSA/*S. flexneri* 6 O-SPC-F3, **4:** BSA/*S. flexneri* 6 O-SPC-F4, **5:** BSA/*S. dysenteriae* type 1 O-SPC-F2, **6:** BSA/*S. dysenteriae* type 1 O-SPC-F3, **7:** BSA/*S. dysenteriae* type 1 O-SPC-F4. A conjugate of full length *S. flexneri* 6 O-SP bound to BSA was prepared as described for *S. flexneri* 2a [9].

Reactivity of *O*-(3-thiopropyl)hydroxylamine with *O*-acetyl groups of *O*-SP

This study was conducted by 1D ^1H NMR at 500 MHz by using a Bruker DRX-500 spectrometer equipped with a 5 mm TXI cryoprobe. A spectral width of 11 ppm was used, together with 32,768 point data sets, forward linear predicted to 65,536 points, a 30° pulse (2.7 μs), and a recycle time of 6 s. *S. flexneri* 6 *O*-SP (5.3 mg) was dissolved in 0.5 mL of 99.96 atom% D_2O , pH was 6.0, and an initial ^1H spectrum was run to define the OAc/NAc pattern. The Ac resonances fell into two groups: 2.196, 2.172, 2.159, 2.156, and 2.149 ppm, which are assigned as OAc, and 2.085, 2.079, 2.074, 2.031, and 2.027 ppm which are assumed to be NAc. It was reasoned that if *O*-(3-thiopropyl)hydroxylamine reacted with OAc on the *O*-SP, then signal(s) would disappear from the OAc group and appear in the NAc group, or at least some changes in the relative intensities of the signals would be observed. An aliquot of 1 mg of *O*-(3-thiopropyl)hydroxylamine solution (corresponding to about a 1:1 molar ratio of *O*-Ac groups to *O*-(3-thiopropyl)hydroxylamine, and brought to pH 5.7 to reproduce the conjugation conditions) was added to the *O*-SP solution. ^1H spectra were run at 5–13 min, 31–37 min, 60–66 min, 120–146 min, and 22 h 2 min–22 h 28 min, and the spectra compared with that of the *O*-SP by overlaying the spectra in dual display mode. No change in the relative intensities of the OAc and NAc groups was observed, and no changes in other parts of the spectra were observed that would support an on-going chemical reaction.

Immunization

Five-six weeks-old female NIH Swiss Webster mice were injected s.c. two or three times at two week intervals with 2.5 μg saccharide as a conjugate, in 0.1 mL PBS. Groups of 10 mice were exsanguinated seven days after the second or the third injections [15]. Controls received PBS.

Antibodies

Serum IgG antibodies were measured by ELISA using *S. flexneri* 2a and 6 and *S. dysenteriae* type 1 LPS as coating antigens. The results were computed using an ELISA data processing program provided by the Biostatistics and Information Management Branch, CDC [16]. Antibody levels were calculated relative to a standard, a pool of high antibody level sera induced by the *O*-SP conjugates, assigned a value of 100 EU. A polyclonal rabbit antiserum, obtained by immunizing rabbits with multiple *i.v.* injections of heat-killed *S. flexneri* 2a or *S. dysenteriae* type 1 bacteria and mice serum raised against *S. flexneri* 6, were used in the immunodiffusion assays.

Statistics

Data are presented as GM values of groups of 10 mice. An unpaired *t* test was used to compare GMs between groups.

Results

Fragments of core and core with one or several RUs for each *Shigella* type separated by gel chromatography are shown in Fig. 1.

Structures of the core fragments

The inner part of the core of all compounds had the common enterobacterial structure (Kdo +3Hep, or Kdo+3Hep+GlcN). Several variants differing in phosphorylation of O-4 of Hep F and in the presence of GlcN N on O-7 of Hep G were observed (Fig. 2). As published for *E. coli* [17], Hep F was phosphorylated when GlcN N was absent (*S. dysenteriae* type 1 and *S. flexneri* 2a) or Hep G was substituted with GlcN N when Hep F had no phosphate (*S.*

flexneri 6). In *S. sonnei* both the GlcN on Hep G and the phosphate on Hep F O-4 were described [8,18]. NMR spectral assignments for the inner part of the core were in agreement with published data for related structures, and are not shown here. The ^1H and ^{13}C signal assignments of the outer part of the cores are shown in Tables 1–3. Monosaccharides were identified on the basis of vicinal proton coupling constants and ^{13}C NMR chemical shifts. Anomeric configurations were deduced from the $J_{1,2}$ coupling constants, from the chemical shifts of H-1 and C-1, C-3 and C-5 signals, and from the intraresidual NOEs. Linkages between monosaccharides were identified on the basis of NOE and HMBC correlations. In addition to transglycosidic NOE correlations between H-1 and a proton at the linkage site, all cores showed correlations from the M1 and L1 protons to Glc H H-4, indicating tight packing of the outer part of the core. The core of *S. flexneri* 2a conformed to that of the *E. coli* R3 type, *S. flexneri* 6 to the R1 type and *S. dysenteriae* type 1 to the R4 type.

Structure and immunogenicity of core and one O-SP RU (core-1RU)

In all cases, the RU was attached to the core by its aminosugar (β -GlcNAc in *S. dysenteriae* type 1 and *S. flexneri* 2a, and β -GalNAc in *S. flexneri* 6). Mass spectroscopy analyses of all compounds showed significant heterogeneity due to variable phosphorylation, acetylation, and the presence of EtN, GlcN (N) side chain, and anhydro-Kdo.

***S. flexneri* 2a**—Assignment of the *S. flexneri* 2a core with one RU spectrum (F4, Figure 1) revealed the attachment of the O-chain to the O-4 of core Glc L. This was confirmed by the results of methylation analysis, which showed the presence of 2,4-disubstituted Glc; all other expected methylated sugars were also identified. The lack of a Glc side chain in the first RU was also confirmed [19,20]. A terminal Rha was present in four variants: non-acetylated, and mono-acetylated at O-2, O-3 or O-4 (Fig. 3). Acetylation was deduced from the downfield shift of the proton signal at the acetylation site. No acetylation of GlcNAc X was observed. Another difference of the first RU from the following RUs, was that GlcNAc was partly acetylated at O-6. ESI-MS of the core-1RU showed the main species having a mass of 2511 amu, corresponding to $\text{Rha}_3\text{Hex}_4\text{Hep}_3\text{GlcN}_2\text{Kdo}_1\text{P}_2\text{Ac}_3$. Higher mass ions were observed at 2553 amu (+EtN or Ac), 2591.4, and 2634.6 amu.

This fraction (core-1RU) conjugated to BSA at an average of 10 saccharide chains per protein, did not react with rabbit anti-*S. flexneri* 2a serum by immunodiffusion, and was not used for mice immunization.

***S. flexneri* 6**—Complete assignment of core-1RU for *S. flexneri* 6 is presented in Table 2. Attachment of the RU GlcNAc X to core O-3 of Glc P followed from NOE data, and was further confirmed by methylation analyses, where the terminal Glc of the core was replaced by 3-substituted Glc in the core-1RU. As in *S. flexneri* 2a, the terminal Rha residue was present in four variants: non-acetylated, and mono-acetylated at O-2, O-3 or O-4 (Fig. 3). The most intense peak in the mass spectrum corresponded to $\text{Rha}_2\text{Hex}_5\text{Hep}_3\text{GalA}_1\text{GlcN}_2\text{Kdo}_1\text{P}_1\text{Ac}_2$ with 2582.4 amu, and a second one to $+\text{PEtN} = 2702.8$ amu. In this spectrum, one Ac is *N*-acetyl group of GalNAc (X) and another is *O*-acetyl group of Rha (T); a minor peak of non-*O*-acetylated variant was also observed at 2540.1 amu.

This fraction (core-1RU) bound to BSA at an average of 12 saccharide chains per BSA molecule, reacted with anti-*S. flexneri* type 6 serum by immunodiffusion, but induced only low levels of anti-LPS antibodies in mice (Table 5).

***S. dysenteriae* type 1**—Assignment of core-1RU of *S. dysenteriae* type 1 is presented in Table 3. The first RU was identical to the following RUs with the exception that GlcNAc

was bound to the core in the β -configuration, while in all other RUs it was present in the α -configuration. The attachment of the first O-SP β -GlcNAc to O-6 of core Gal followed from NOE and HMBC data, and was confirmed by methylation analysis.

Core-1RU bound to BSA at an average of 23 oligosaccharide chains per protein reacted with anti- *S. dysenteriae* type 1 serum by immunodiffusion, and when injected into mice induced LPS antibodies (Table 6).

Structure and immunogenicity of core and several RUs (O-SPC)

Longer fractions (F2 and F3, Figure 1) were too heterogeneous in size and substitutions to interpret their mass spectra, therefore integration of signals in ^1H -NMR spectra was used to calculate an average number of O-SP repeats in an O-SPC chain.

***S. flexneri* 2a**—For F2 and F3, integrations of the H-6 proton signals for Rha methyl groups in ^1H -NMR spectra (1.16–1.31 ppm) relative to the anomeric signals of core α -Gal K (5.90 ppm) and α -Glc L (5.52 ppm) were used to calculate the average number of O-SP repeats (Fig. 4). F3 contained core plus an average of 2 RU and F2 contained core with an average of 3 RU (Figure 1). Both fractions were bound to BSA at an average of 8 chains per BSA molecule. These conjugates reacted with anti-*S. flexneri* 2a rabbit serum by immunodiffusion and induced LPS antibodies in mice with booster responses (Table 4).

***S. flexneri* 6**—Similarly to *S. flexneri* 2a, integrations of the Rha methyl signals in ^1H -NMR spectra (1.16–1.30 ppm) relative to the anomeric signals of core α -Glc K (5.84 ppm) and α -Gal L (5.62 ppm) were used to calculate the average number of O-SP repeats. F3 contained an average of 2.5 RU and was bound to BSA at an average of 9 chains per BSA molecule (Table 5). This conjugate was antigenic, but similarly to the F4 conjugate (core-1RU) was poorly immunogenic. The F2 conjugate composed of about 7 RU plus core bound at an average density of 3.5 chains per protein was a better immunogen ($p < 0.01$). It was not more immunogenic than the full length O-SP bound to BSA by multiple attachments using ADH as a linker (Table 5).

***S. dysenteriae* type 1**—To estimate the number of RU in *S. dysenteriae* type 1 fractions, integration of the anomeric signal of the terminal Rha T (5.08 ppm) relative to the substituted T (5.11 ppm) was used (Fig. 5). F2 contained core plus an average of 3 RUs, and F3 - 2 RUs. 2D NMR of F3 confirmed the β -configuration (H/C-1: 4.65/102.1; H/C-2: 3.84/55.3 ppm) of GlcNAc in the first RU, linked to core. The GlcNAc in all the following RUs of the O-SP was in the α -configuration (H/C-1: 5.05/95.2; H/C-2: 4.15/53.2 ppm). Separate signals for Gal Y, linked to β - (in the first RU) and α -GlcNAc (in the second RU) were also observed with corresponding NOE correlations to H-3 of GlcNAc. Both fractions bound to BSA were antigenic and induced similar antibody levels in mice (Table 6).

Discussion

The LPS of Gram-negative bacteria may be present in three forms: “rough” consisting of Lipid A and a core only, “semi-rough” consisting of Lipid A, core and a small number of O-SP RUs (O-SPC), or as “smooth” with 10–20 RUs (O-SP). The LPS molecule can also undergo bacteriophage-encoded modification like glucosylation or *O*-acetylation [19]. Most of the *S. flexneri* LPS cores conformed to the *E. coli* R3 type [21,22]. We confirmed the presence of R3 type core in *S. flexneri* 2a and demonstrated the linkage and the unique structure (lack of Glc side chain and the pattern of *O*-acetylation) of the first O-SP RU (Figure 2). The structure of the *S. flexneri* 6 core, including the saccharide to which the O-chain is attached, but without indication of the exact attachment position, has been reported

[23]. Now, we have confirmed that the *S. flexneri* 6 core conforms to the *E. coli* R1 type and have shown that the O-SP is attached to the core through its β -GalNAc (Figure 2). The core of *S. dysenteriae* type 1 conforms to that of *E. coli* R4 type. The first O-SP RU was identical to the other RUs, with the exception that the GlcNAc was bound to the core in the β -configuration, while in all other RUs, the GlcNAc was present in the α -configuration [24]. For *S. sonnei*, the structure of the LPS R1 type core and the linkage of the O-SP have been reported [18].

Investigational vaccines composed of protein conjugates of the O-SP of *S. dysenteriae* type 1, *S. sonnei*, and *S. flexneri* 2a were safe and elicited specific LPS antibodies in young adults [9]. The *S. sonnei* O-SP conjugate had > 70% efficacy in Israeli army recruits and in children older than three years of age [3,6]. As shigellosis peaks in 1–4 year old children, a more immunogenic vaccine is needed. The immunogenicity of *S. dysenteriae* type 1 and *S. sonnei* conjugates was significantly improved by using low molecular mass saccharides, whether synthesized or isolated from LPS, bound by their reducing ends to protein carriers at defined densities [7,8]. Now, we examined if a similar approach could be adapted to other *Shigella* LPS. The first RU of *S. flexneri* 2a O-SP was different from the following RUs of the O-SP because it lacked a Glc side chain, there was no *O*-Ac on GlcNAc, and its non-reducing terminal Rha was heterogeneously *O*-acetylated at all positions [25,26]. Probably, because of these modifications, core-1RU conjugated to protein was not antigenic using anti-LPS serum. Longer O-SPC with an average 2 or 3 O-SP RUs were antigenic and induced LPS antibodies in mice with booster responses. Antibody levels were comparable, but not higher than those induced by O-SP conjugates used in clinical trials [9]. Synthetic protein-carbohydrate conjugates of *S. flexneri* 2a O-SP fragments were shown to be immunogenic in mice but it is difficult to compare antibody levels between laboratories [27]. Similar to *S. flexneri* 2a, the *O*-acetylation of the first RU of *S. flexneri* 6 was different from the following RUs: the terminal Rha was heterogeneously *O*-acetylated at all positions, while in the latter RUs this Rha was reported to be *O*-acetylated on position O-3 only [28]. That could be part of the reason why O-SPC with an average of 1 or 2 RU induced only low antibody levels. Conjugates of O-SPC with an average of 7 RUs and of full length O-SP induced similar and higher antibody levels than those containing 1 or 2 RUs. In the case of *S. dysenteriae* type 1, all RUs were identical with the exception of the β -configuration of the GlcNAc bound to the core (the α -configuration is present in all other RUs). In this case, the core with even 1 RU was antigenic, and also immunogenic in mice. Antibody levels induced by these conjugates containing 1, 2 or 3 RU were similar to those of the synthetic saccharide conjugates, and statistically higher than those induced by the O-SP conjugates [7]. Tailoring of these conjugates to produce the most immunogenic constructs and characterization of the epitopes recognized by the O-SPC induced antibodies are planned.

The O-SPC conjugates, resembling synthetic conjugates, induced similar (*S. flexnerii* 2a and 6) or higher (*S. sonnei* and *S. dysenteriae* type 1) antibody levels in mice than the full length O-SP conjugates. They are easy to prepare, characterize and standardize. The conjugation method can be applied to other Gram-negative bacteria.

Abbreviations

COSY	correlation spectroscopy
TOCSY	total correlation spectroscopy
NOESY	nuclear Overhauser enhancement spectroscopy
HSQC	heteronuclear single quantum coherence

HMBC	heteronuclear multiple bond connectivity
LPS	lipopolysaccharide
O-SP	O-specific polysaccharide
O-SPC	low molecular mass O-SP-core fragments
GlcN	glucosamine
GlcNAc	N-acetylglucosamine
Hep	<i>L-glycero-D-manno</i> -heptose
Kdo	3-deoxy-D- <i>manno</i> -octulosonic acid
RU	repeat unit of O-SP
ES-MS	electrospray mass spectrometry
GLC-MS	gas liquid chromatography mass spectrometry
GM	geometric mean
SBAP	<i>N</i> -succinimidoyl 3-(bromoacetamido) propionate
TFA	trifluoroacetic acid
PBS	phosphate buffer saline, pH 7.4
s.c.	subcutaneously
EU	Elisa units
GM	geometric mean

Acknowledgments

This research was supported by the Intramural Research Program of the NIH, NICHD. The authors thank Chunyan Guo for technical assistance.

References

1. Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ, Adak GK, Levine MM. Bull. World Health Organ. 1999; 77:651–666. [PubMed: 10516787]
2. Levine MM, Kotloff KL, Barry EM, Pasetti MF, Sztein MB. Nat. Rev. Microbiol. 2007; 5:540–553. [PubMed: 17558427]
3. Passwell JH, Ashkenazi S, Banet-Levi Y, Ramon-Saraf R, Farzam N, Lerner-Geva L, Even-Nir H, Yerushalmi B, Chu C, Shiloach J, Robbins JB, Schneerson R. Vaccine. 2010; 28:2231–2235. [PubMed: 20056180]
4. Guerrant RL, Kirchhoff LV, Shields DS, Nations MK, Leslie J, de Sousa MA, Araujo JG, Correia LL, Sauer KT, McClelland KE. J. Infect. Dis. 1983; 148:986–997. [PubMed: 6361176]
5. Noriega FR, Liao FM, Maneval DR, Ren S, Formal SB, Levine MM. Infect. Immun. 1999; 67:782–788. [PubMed: 9916090]
6. Cohen D, Ashkenazi S, Green MS, Gdalevich M, Robin G, Slepon R, Yavzori M, Orr N, Block C, Ashkenazi I, Shemer J, Taylor DN, Hale TL, Sadoff JC, Pavliakova D, Schneerson R, Robbins JB. Lancet. 1997; 349:155–159. [PubMed: 9111538]
7. Pozsgay V, Chu C, Pannell L, Wolfe J, Robbins JB, Schneerson R. Proc. Natl. Acad. Sci. U. S. A. 1999; 96:5194–5197. [PubMed: 10220442]
8. Robbins JB, Kubler-Kielb J, Vinogradov E, Mocca C, Pozsgay V, Shiloach J, Schneerson R. Proc. Natl. Acad. Sci. U. S. A. 2009; 106:7974–7978. [PubMed: 19346477]

9. Taylor DN, Trofa AC, Sadoff J, Chu C, Bryla D, Shiloach J, Cohen D, Ashkenazi S, Lerman Y, Egan W, Schneerson R, Robbins JB. *Infect. Immun.* 1993; 61:3678–3687. [PubMed: 8359890]
10. Westphal O, Jann K. *Methods Carbohydr. Chem.* 1965; 5:83–91.
11. Peterson GL. *Anal. Biochem.* 1979; 100:201–220. [PubMed: 393128]
12. Bailey RW. *Biochem. J.* 1958; 68:669–672. [PubMed: 13522678]
13. Ciucanu I, Caprita R. *Anal. Chim. Acta.* 2007; 585:81–85. [PubMed: 17386650]
14. Kubler-Kielb J, Pozsgay V. *J. Org. Chem.* 2005; 70:6987–6990. [PubMed: 16095333]
15. Schneerson R, Barrera O, Sutton A, Robbins JB. *J. Exp. Med.* 1980; 152:361–376. [PubMed: 6967514]
16. Plikaytis, BD.; Carlone, GM. *Program ELISA for Windows User's Manual.* v. 2. Atlanta, GA: Centers for Disease Control; 2005.
17. Muller-Loennies S, Lindner B, Brade H. *Eur. J. Biochem.* 2002; 269:5982–5991. [PubMed: 12444988]
18. Gamian A, Romanowska E. *Eur. J. Biochem.* 1982; 129:105–109. [PubMed: 6761115]
19. West NP, Sansonetti P, Mounier J, Exley RM, Parsot C, Guadagnini S, Prevost MC, Prochnicka-Chalufour A, Delepierre M, Tanguy M, Tang CM. *Science.* 2005; 307:1313–1317. [PubMed: 15731456]
20. Takeshita M, Makela PH. *J. Biol. Chem.* 1971; 246:3920–3927. [PubMed: 4104709]
21. Jansson PE, Lindberg B, Lindberg AA, Wollin R. *Carbohydr. Res.* 1979; 68:385–389. [PubMed: 371805]
22. Amor K, Heinrichs DE, Fridrich E, Ziebell K, Johnson RP, Whitfield C. *Infect. Immun.* 2000; 68:1116–1124. [PubMed: 10678915]
23. Lugowski C, Kulakowska M, Romanowska E. *J. Immunol. Methods.* 1986; 95:187–194. [PubMed: 3098854]
24. Falt IC, Lindberg AA. *Microb. Pathog.* 1994; 16:27–41. [PubMed: 7520112]
25. Kubler-Kielb J, Vinogradov E, Chu C, Schneerson R. *Carbohydr. Res.* 2007; 342:643–647. [PubMed: 17087926]
26. Perepelov AV, L'vov VL, Liu B, Senchenkova SN, Shekht ME, Shashkov AS, Feng L, Aparin PG, Wang L, Knirel YA. *Carbohydr. Res.* 2009; 344:687–692. [PubMed: 19246033]
27. Phalipon A, Tanguy M, Grandjean C, Guerreiro C, Belot F, Cohen D, Sansonetti PJ, Mulard LA. *J. Immunol.* 2009; 182:2241–2247. [PubMed: 19201878]
28. Dmitriev BA, Knirel YA, Sheremet OK, Shashkov AS, Kochetkov NK, Hofman IL. *Eur. J. Biochem.* 1979; 98:309–316. [PubMed: 381001]

Figure 1.

BioGel P-10 gel filtration profiles of 2% acetic acid hydrolyzed *Shigellae* LPSs. F1 – O-SP; more than 10 RU; F2, F3 – core + avr. 2–5 O-SP RU; F4 – core + avr. 1 O-SP RU.

Shigella flexneri 2a core (type R3) with one RU

Rha T is presented in 4 variants, with no OAc (T^0), 2-OAc (T^2), 3-OAc (T^3), 4-OAc (T^4), 3~2~0>4.

Shigella flexneri 6 core (type R1) with one RU

Rha T is presented in 4 variants, with no OAc (T^0), 2-OAc (T^2), 3-OAc (T^3), 4-OAc (T^4), 3>2>0>4.

Shigella dysenteriae type 1 core (type R4) with one RU **Shigella sonnei core (type R1) with one RU [8,18]**

GlcN N is ~50% present

Figure 2.

Structures of *Shigellae* O-SPC fragments of core plus one O-SP repeat unit (RU).

Figure 3. Fragment of COSY (green-cyan) and TOCSY (red) spectra of the core-RU from the LPSs of *S. flexneri* O2a (top) and *S. flexneri* O6 (bottom), illustrating *O*-acetylation of the terminal rhamnose residues (T). Proton signals at the acetylation site are shifted to low field.

Figure 4.

Integration of the proton signals of *S.flexneri* 2a 1 O-SPC fractions for the determination of the average number of O-SP repeat units (RU). Rha methyl signals in ^1H -NMR spectra (1.16–1.31 ppm) in the O-SP relative to the anomeric signals of the core α -Gal K (5.90 ppm) and α -Glc L (5.52 ppm) were used. F1-O-SP; F2-core-3RU; F3-core-2RU; F4-core-1RU; F5-core.

Figure 5.

Integration of the anomeric signals of *S. dysenteriae* 1 O-SPC fractions for the determination of the average number of O-SP repeat units (RU). The anomeric signal of the terminal Rha T (5.08 ppm) relative to that of the substituted Rha T (5.11 ppm) was used. F1-O-SP; F2-core-3RU; F3-core-2RU; F4-core-1RU.

Table 1

NMR data for *S. flexneri* 2a core plus one O-SP repeat unit.

Unit		1	2	3	4	5	6a/b
Rha T ⁰ no Ac	H	4.96	4.08	3.80	3.44	3.70	1.26
	C	103.5	71.3	71.7	73.1	70.3	17.8
Rha T ² 2-O-Ac	H	5.01	5.24	3.99	3.49	3.78	1.29
	C	100.4	73.5	70.7	73.4	70.4	17.8
Rha T ³ 3-O-Ac	H	4.98	4.23	5.00	3.65	3.82	1.29
	C	103.3	71.0	74.7	72.4	70.4	17.8
Rha T ⁴ 4-O-Ac	H	5.01	4.13	4.01	4.89	3.87	1.16
	C	103.3	71.2	70.7	75.3	68.1	17.8
Rha Z	H	5.16	4.06	3.92	3.49	3.73	1.31
	C	102.0	79.2	71.2	73.3	70.4	17.9
Rha Y	H	4.84	3.82	3.79	3.55	4.03	1.25
	C	102.7	71.8	78.2	72.9	70.3	17.6
GlcNAc X	H	4.59	3.95	3.62	3.57	3.49	3.75/3.94
	C	102.5	56.7	83.2	69.6	77.3	61.7
Glc L	H	5.52	3.79	4.09	3.57	3.80	3.71/3.87
	C	91.6	75.6	72.0	81.4	73.4	61.8
Glc M	H	5.21	3.55	3.75	3.45	3.99	3.75/3.80
	C	97.2	70.8	74.5	70.6	73.1	61.8
Gal K	H	5.90	4.11	4.25	4.30	4.29	3.77/3.79
	C	95.1	68.7	71.2	65.3	71.1	61.7
GlcNAc P	¹ H	5.11	4.02	3.80	3.57	4.05	3.79/3.93
	¹³ C	93.5	54.7	72.3	71.3	73.2	61.9
Glc H	H	5.20	3.68	4.14	3.82	3.90	3.78/3.89
	C	102.5	71.4	76.2	71.8	73.5	60.8

Table 2

NMR data for *S. flexneri* 6 core plus one O-SP repeat unit.

Unit	Atom	1	2	3	4	5	6 (a/b)
Rha T ⁰ no Ac	H	4.96	4.06	3.88	3.43	3.76	1.26
	C	103.3	71.3	71.7	73.4	70.4	17.8
Rha T ² 2-O-Ac	H	5.02	5.22	3.97	3.49	3.48	1.29
	C	100.5	73.6	70.7	73.4	70.4	17.8
Rha T ³ 3-O-Ac	H	4.98	4.20	4.99	3.64	3.82	1.30
	C	103.0	69.4	74.7	73.4	70.4	17.8
Rha T ⁴ 4-O-Ac	H	5.00	4.11	3.99	4.88	3.87	1.16
	C	103.2	71.2	70.7	75.3	68.1	17.8
Rha Z	H	5.37-5.42	4.08-4.10	3.88	3.44;3.49*	3.65	1.26
	C	101.0	80.9	70.9	73.2	70.6	17.8
Gal A Y	H	4.51	3.63	3.84	4.35	4.33	
	C	105.7	71.1	74.0	77.4	74.5	172.0
GalNAc X	H	4.79	4.08	3.91	4.26	3.74	3.83/3.83
	C	103.0	52.9	81.6	69.0	76.1	62.3
Gal L	H	5.62	3.99	4.22	4.01	4.16	3.74/3.74
	C	92.6	73.6	69.2	70.5	72.5	62.6
Gal M	H	5.33	3.86	4.00	3.96	4.16	3.74/3.74
	C	97.0	69.5	70.5	70.7	72.5	62.6
Glc K	H	5.84	3.89	4.20	3.59	4.12	3.81/3.87
	C	95.8	73.7	79.1	69.0	72.5	61.7
Glc H	H	5.28	3.69	4.06	3.80	3.86	3.86/3.91
	C	101.7	71.7	77.5	71.7	73.7	61.2
Glc P	H	4.76	3.42	3.74	3.53	3.47	3.75/3.92
	C	103.6	73.9	86.0	69.6	76.6	62.0

Table 3

NMR data for *S. dysenteriae* type 1 core plus one O-SP repeat unit.

Unit		1	2	3	4	5	6a/b
Rha T	H	5.08	4.07	3.85	3.47	3.85	1.32
	C	103.5	71.4	71.4	73.3	70.3	17.8
Rha Z	H	5.06	4.17	3.87	3.56	3.88	1.32
	C	102.8	70.8	79.2	72.6	70.5	17.8
Gal Y	H	5.60	3.95	3.93	4.10	3.90	3.77/3.77
	C	98.7	75.0	70.1	70.5	72.2	61.8
GlcNAc X	H	4.65	3.84	3.84	3.71	3.49	3.77/3.93
	C	102.2	55.3	78.4	72.6	77.0	61.7
Gal P	H	4.47	3.56	3.65	3.91	3.81	3.89/3.96
	C	104.3	72.1	73.6	69.3	74.0	68.5
Gal M	H	5.27	3.86	3.97	4.02	4.17	3.76/3.76
	C	97.1	69.5	70.6	71.0	72.5	62.5
Gal L	H	5.58	4.02	4.12	4.07	4.10	3.77/3.77
	C	93.7	73.5	69.0	70.5	72.4	62.4
Glc K	H	5.76	3.78	4.03	3.73	4.18	3.88/4.01
	C	96.1	75.1	71.2	79.3	71.3	60.9
Glc H	H	5.21	3.68	4.08	3.77	3.92	3.82/3.89
	C	102.6	71.4	77.8	71.5	73.6	61.0

Table 4

Composition and Geometric Mean of serum IgG anti-*S. flexneri* 2a LPS induced by O-SPC bound to BSA.

Conjugate	Mol. Mass of conjugate [kDa] ¹	Mol. mass of O-SPC [Da]	No of chains per BSA	BSA: Sugar ratio [wt:wt]	IgG anti-LPS [EU] ²	
					2 nd inj.	3 rd inj.
BSA/F2: core + 3 RU ³	103	4202	8	1:0.5	0.1	10
BSA/F3: core + 2 RU	101	3356	8	1:0.5	0.2	11

¹ EU – ELISA units.

² Mass of O-SPC is calculated assuming one Glc per RU, except the first one, and one *O*-acetyl group. The number of O-SP repeat units (RU) is an average for the given fraction.

Mice (10 per group) were injected with 2.5 µg of saccharide as a conjugate per mouse, 2 or 3 times, 2 weeks apart and bled one week after the last two injections.

Table 5

Composition and Geometric Mean of serum IgG anti-*S. flexneri* 6 LPS induced by O-SP and O-SPC bound to BSA.

Conjugate	Mol. mass of conjugate [kDa]	Mol. mass of O-SPC [Da]	No of chains per BSA	BSA: Sugar ratio [wt:wt]	IgG anti-LPS [EU]/
BSA/F1: core + 15–20 RU ²	na ³	>10000	na	1:0.4	21.3
BSA/F2: core + 7 RU	96	6607	3.5	1:0.4	23.7
BSA/F3: core + 2.5 RU	106	3588	9	1:0.5	1.6
BSA/F4: core + 1 RU	106	2581	12	1:0.5	1.2

/ EU – ELISA units.

²The number of O-SP repeat units (RU) is an average for the given fraction.

³na – not analyzed.

Mice (10 per group) were injected with 2.5 µg of saccharide as a conjugate per mouse, 3 times, 2 weeks apart and bled one week after the 3rd injection.

Table 6

Composition and Geometric Mean of serum IgG anti-*S. dysenteriae* type 1 LPS induced by O-SPC conjugates bound to BSA.

Conjugate	Mol. mass of conjugate [kDa]	Mol. Mass of O-SPC [Da]	No of chains per BSA	BSA: Sugar ratio [wt:wt]	IgG anti-LPS [EU]/
BSA/F2: core + 3 RU ²	130	3550	16	1:0.9	10.0
BSA/F3: core + 2 RU	133	2892	21	1:0.9	7.6
BSA/F4: core + 1 RU	125	2235	23	1:0.8	21.9

¹ EU – ELISA units.

² The number of O-SP repeat units (RU) is an average for the given fraction.

³ na – not analyzed.

Mice (10 per group) were injected with 2.5 µg of saccharide as a conjugate per mouse, 3 times, 2 weeks apart and bled one week after the 3rd injection.