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Conjugation of meningococcal lipooligosaccharides through their lipid A terminus conserves their inner epitopes and results in conjugate vaccines having improved immunological properties

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Abstract

The importance of conserved inner saccharide epitopes to the immune performance of meningococcal lipooligosaccharide–protein conjugate vaccines was demonstrated in the following experiments. Two different oligosaccharides were obtained by chemical degradations of the same L7 lipooligosaccharide, and both were linked terminally to tetanus toxoid. One was a truncated oligosaccharide in which the inner epitopes were incomplete and was obtained by mild acid hydrolysis of the L7 lipooligosaccharide. This oligosaccharide was conjugated by direct reductive amination through its newly exposed terminal Kdo residue. The second, a full-length oligosaccharide, was obtained by O-deacylation of the L7 lipooligosaccharide, with subsequent removal of phosphate substituents from its lipid A moiety using alkaline phosphatase. This permitted the full-length oligosaccharide to be conjugated directly to tetanus toxoid by reductive amination through its newly exposed terminal 2-*N*-acyl-2-deoxy-D-glucopyranose residue. Comparison of the immune performance of the two conjugates in mice revealed, that while both were able to induce significant levels of L7-lipooligosaccharide-specific IgG antibody, the conjugate made with the full-length saccharide was able to induce antibodies with increased bactericidal activity against homologous meningococci.

Keywords: Meningococcal lipooligosaccharides; Conjugate vaccines; Immunological properties

1. Introduction

Neisseria meningitidis is a human pathogen of worldwide significance. Despite the success of current vaccines composed of groups A, C, W-135 and Y capsular polysaccharides, and the more recent improved group C polysaccharide conjugate vaccines,[1] the group B polysaccharide is precluded from the above vaccines, even though it is a major contributor to the burden of disease in developed countries.[2] This is because of the poor immunogenicity of the group B polysaccharide in both its native[3] and conjugated forms.[4,5] Consequently alternative vaccines based on subcapsular antigens, including lipooligosaccharides (LOS), are being explored.

The meningococcal LOS have been implicated in the immune response to natural infection,[6,7] but their use as vaccines is contraindicated because of their high toxicity. They also exhibit considerable antigenic diversity, which also remains a major challenge. Currently there are 12 known different immunotypes,[8–11] of which types L1–L7 are exclusively associated with groups B and C meningococci, and types L10–L12 with group A meningococci. Only types L8 and L9 overlap between the two groups. The epitopes responsible for the immunotyping are located in the oligosaccharide moieties of the LOS,[12] which have been shown to be structurally diverse,[13–18] as well as having some regions of similarity.

To avoid the toxicity of the LOS, the toxic lipid A moiety can be removed by mild acid hydrolysis, and subsequently the innocuous oligosaccharides can be conjugated by different methods to protein carriers through their terminal 2-keto-3-deoxyoctulosonic acid

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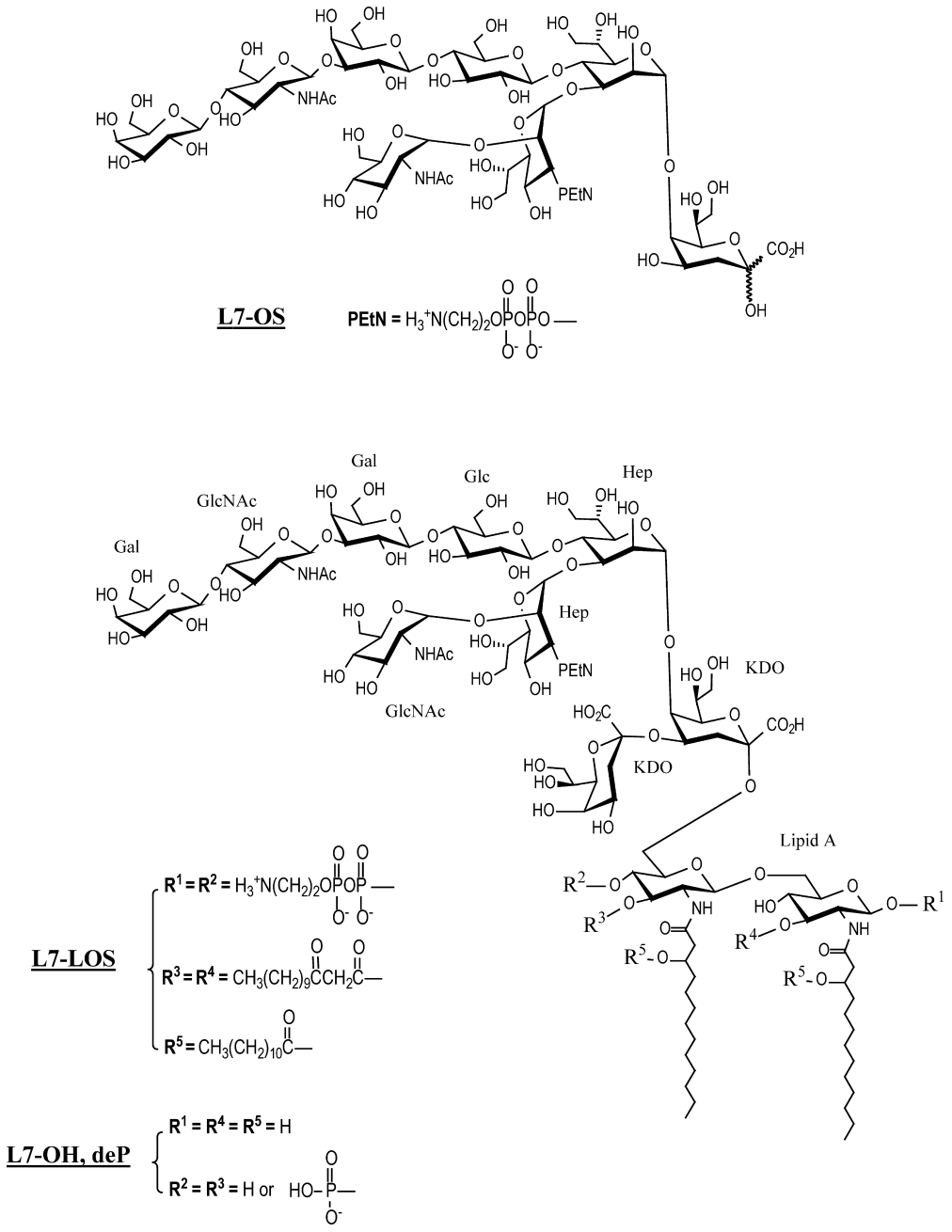

Fig. 1. Structures of the truncated (L7-OS) and full-length (L7-OH and de P) oligosaccharides prior to conjugation.

(Kdo) residues.[12,19,20] Although L10 conjugates were able to induce in mice oligosaccharide-specific antibodies that were bactericidal,[12,19] conjugates made with oligosaccharides associated with groups B and C meningococci in comparison produced antisera with sub-optimal bactericidal activity.[12,20] This was particularly noticeable in the case of the L3 and L7 immunotypes, which are unfortunately the most prevalent among groups B and C meningococcal isolates.[9] Both the L3 and L7 immunotypes have similar structures, shown in Fig. 1. L7 is simply the desialylated form of L3.[18]

One possible reason for the sub-optimal immune performance of the above conjugates is that the point of cleavage of the LOS, at the Kdo residue, is close to if not within the internal oligosaccharide epitopes, thus impairing their structures. The importance of internal epitopes to the immune response has been documented[21,22] and is due to structural similarity between the nonreducing distal oligosaccharide chain of the LOS and mammalian tissue antigens, which results in immunodominance of the internal epitopes.[15,21,23–25]

To test this hypothesis, we compared the immune response of two L7-LOS–TT conjugates. One was

Table 1
^{31}P NMR data obtained with L7-LOS and its modified products

L7-LOS and treatment with reagents	^{31}P Chemical Shift Values [a] (ppm)						
	Pyrophosphoryl-ethanolamine		Pyrophosphomonoester	Phosphoryl-ethanolamine	Monophosphate		
	C-4′	C-1	C-1, C-4	C-3 (Hep)	C-4′	C-1	C-3′
L7-LOS	−9.53(1.0)	−10.36(1.4)	−5.21(0.3)	+0.70(1.0)	+5.21(0.3)	+2.77(0.2)	[b]
Hydrazine	−9.59(0.5)	−10.14(0.4)		+0.73(1.0)	+5.00(0.4)		
Alkaline-phosphatase[1]				+0.76(1.0)	+5.21(0.5) [d]		+3.4(0.3) [c]
Alkaline-phosphatase[2]				+0.73(1.0)	+5.20(0.6)		+3.4(0.6) [c]

[a] Values relative to the external standard H_3PO_4.
[b] Not detected.
[c] Tentative assignment.
[d] Cross-peak with H-4′ (HSQC experiment).

Table 2
Composition of L7-OS-TT and L7-OH, de P-TT conjugates

Conjugate	% Saccharide [a]	% TT [b]	Average molar ratio Saccharide/TT [c]
L7-OS-TT	20.0	79.0	18.9:1
L7-OH, de P-TT	26.2	72.5	15.5:1

[a] Estimated by phenol–sulfuric assay using the appropriate saccharides as standards.
[b] Protein content was estimated by the bicinchoninic acid assay using TT as the standard.
[c] The molar ratio of saccharide/TT was calculated using molecular weights of 1590 for L7-OS, 2534 for L7-OH, de P and 150,000 for TT.

made with terminally linked, hydrazine-treated, full-length oligosaccharide (L7-OH, de P), and the other with a similarly linked but truncated oligosaccharide (L7-OS).

2. Results

2.1. Characterization of oligosaccharides

As previously described,[12,18] application of a 1% HOAc hydrolysate of the L7-LOS to a Bio-Gel P4 column yielded an oligosaccharide (L7-OS), whose structure, shown in Fig. 1, was confirmed by ^{1}H NMR spectroscopy.[18]

The L7-LOS was also O-deacylated using anhydrous hydrazine,[18] prior to being further dephosphorylated with alkaline phosphatase, to yield L7-OH, de P. In addition to O-deacylation, the structural changes wrought by each of the above procedures were monitored by ^{31}P NMR spectroscopy, resulting in the determination of the structure of the oligosaccharide (L7-OH, de P) used for conjugation (Fig. 1). The chemical shifts of the ^{31}P NMR signals of the native and modified L7-LOS are listed in Table 1, and assignments were made largely by comparison with other ^{31}P NMR studies on both LOS[18] and lipid A.[26–29]

The native L7-LOS whose structure is depicted in Fig. 1 exhibited two ^{31}P signals at -10.36 ppm and -9.53 ppm, which were assigned to diphosphorylethanolamine groups situated at C-1 and C-4′ of the lipid A moiety.[28] Another smaller signal at -5.21 ppm was assigned to a pyrophosphomonoester group also located at C-1 and/or C-4′ of lipid A, which is consistent with previous assignments[28] on the meningococcal LOS and with the conclusion that these positions are not all fully substituted with diphosphorylethanolamine groups.[29] The signal at $+0.70$ ppm was assigned to a monophosphorylethanolamine substituent at O-3 of one of the heptose residues.[18] Treatment of the L7-LOS with hydrazine reduced by half the intensity of the signals at C-1 and C-4′ and eliminated the pyrophosphomonoester signal at -5.21 ppm.[29] It also produced two new signals at $+4.97$ ppm and $+2.53$ ppm, which were assigned to monophosphate esters at C-4′ and C-1, respectively. Thus hydrazine treatment is capable of partially degrading the diphosphorylethanolamine groups to monophosphate esters.

The remaining diphosphorylethanolamine groups at C-1 and C-4′ were completely removed by the first alkaline phosphatase treatment, while the monophosphorylethanolamine phosphate substituent on the heptose residue remained intact. This was ascertained by the disappearance, from the ^{31}P NMR of the alkaline phosphatase-treated product, of the two ^{31}P signals, previously assigned to the former groups, and the retention of the ^{31}P signal previously assigned to the latter (Table 1). Some monophosphate ester remained on C-4′, the assignment of which was confirmed by a 2D hetero-correlated HSQC experiment. Some had migrated to C-3′, but none remained on C-1. This indicated that L7-OH, de P was heterogeneous in terms of the presence or absence of the above phosphate groups, but that the hemiacetal group on its terminal glucosamine residues was completely exposed. Therefore, because a second treatment with alkaline phosphatase failed to remove the residual monophosphate ester at C-4′ and C-3′, we decided to conjugate L7-OH, de P in its heterogeneous form.

2.2. Conjugation of oligosaccharides to tetanus toxoid

Oligosaccharides L7-OS and L7-OH, de P were conjugated to tetanus toxoid (TT) using the procedure previously described by Jennings and Lugowski,[5] but with minor modification. This time conjugations were carried out in 0.2 M borate buffer at pH 9.0, which improved the yields of the conjugates. Conjugation was easily monitored by HPLC because the conjugate peak was separated by 8 min from the TT peak and was judged to be complete when the TT peak disappeared. The conjugates were purified by column chromatography, and their stoichiometric analysis is shown in Table 2. Both conjugates had comparable oligosaccharide/TT ratios.

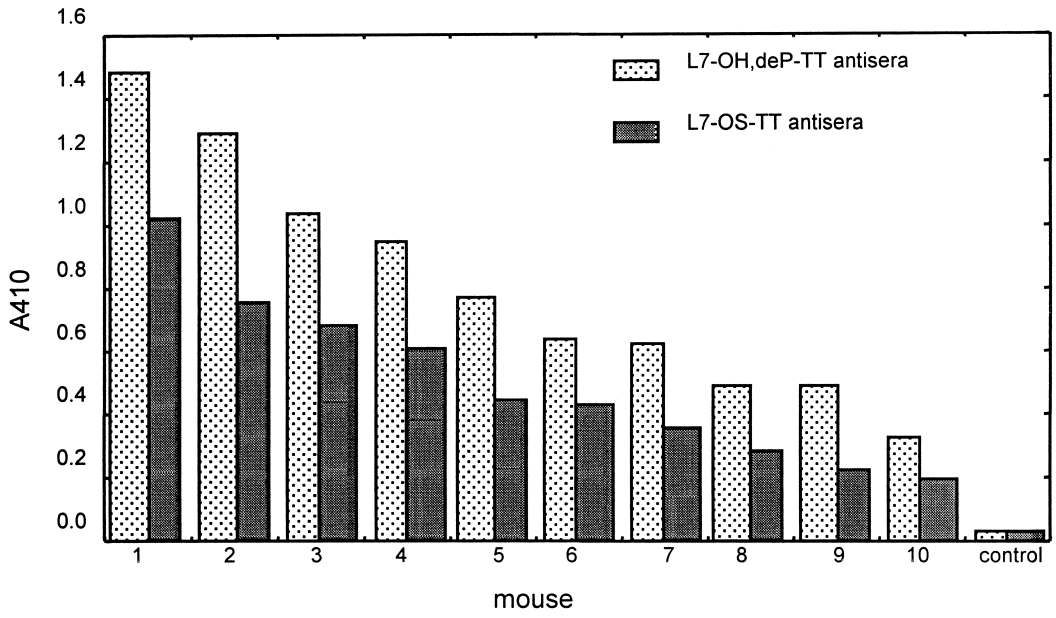

Fig. 2. Comparative ELISA titers of individual mouse antisera following immunization with either L7-OS-TT or L7-OH, de P-TT.[1–10] The antisera from each immongen are paired from 1–10 according to decreasing titer. The titers of two control unimmunized mice are also included in 11. Statistical analysis of results showed that the average increase in antibody titers induced by L7-OH, de P-TT over that induced by L7-OS-TT was significant ($p < 0.04$).

2.3. Immunogenicity of conjugates in mice

The L7-OS-TT and L7-OH, de P-TT conjugates were evaluated for their immunogenicity in mice. Each group of mice was given three subcutaneous injections with vaccines containing 2.5 μg of carbohydrate together with RIBIs complete adjuvant before being bled. The ELISA titers of the individual mice are shown in Fig. 2, using the L7-LOS as the coating antigen, and these show that the preimmune sera contained only trace amounts of L7-LOS-specific antibodies. While both conjugates induced antibody having this latter specificity, the levels induced by L7-OH, de P-TT were consistently higher than those induced by L7-OS-TT. In addition the subclass distribution of antibodies elicited by both conjugates was not significantly different. Both conjugates were able to produce high levels of potentially bactericidal IgG2a, IgG2b and IgG3 antibodies.

2.4. Bactericial activity of antisera

The bactericidal activities of the antisera induced in mice by L7-OS-TT and L7-OH, de P-TT conjugates against the homologous immunotype organism are shown in Fig. 3. Although both conjugates were able to elicit L7-LOS-specific antibodies containing potential bactericidal isotypes, the antisera induced by L7-OH, de P-TT had the greater bactericidal activity.

3. Discussion

In previous studies on LOS conjugate vaccines the problem of the toxicity of the LOS was avoided by removal of the toxic lipid A from the molecule by mild acid hydrolysis.[12,19,23] Conjugates were then made using the non-toxic truncated core oligosaccharides as haptens. A number of different chemical procedures were used in the syntheses of the conjugates but all of them utilized the newly exposed Kdo residue as the linkage point.[12,19,23] The conjugates were evaluated in animals, and some general conclusions can be gleaned from the results obtained. Only conjugates made with immunotypes associated with group A strains (L8–L12) were able to induce antibodies having good bactericidal activity.[12,19] Whereas conjugates made with immunotypes associated with group B and C strains, such as L3 and L7, induced antibodies having no bactericidal activity.[19,23] This was disappointing because L3 and L7, are the most prevalent immunotypes found among group B *N. meningitidis* disease isolates.[9] The ability of the group A associated immunotypes to induce bactericidal antibody is probably structure-based. Unlike the LOS of group B and C strains, which have an immunosuppressive lacto-*N*-neotetraose chain or its sialylated analog attached to a Hep in their inner structures,[15,16,18] the LOS of group A strains have more immunogenic distal chain structures[1]. It is known that the L3 and L7

[1] Unpublished results.

immunotypes are closely related, differing only by the addition of terminal sialic acid to the lacto-*N*-neotetraose chain of the former.[18] Therefore, in addition to the poor immunogenicity of the L3 and L7 immunotypes conferred on them by the above structural features, another factor to consider in their inability to raise bactericidal antibody is that cleavage of the LOS at the internal Kdo residue could result in structural impairment of the LOS inner epitope. To test this hypothesis, we conjugated an L7 oligosaccharide having conserved inner epitopes to TT, and compared the immune properties of the conjugate with those of a TT conjugate made as previously described with a truncated L7 core oligosaccharide. To achieve our objectives we chose to conjugate the hydrazine-treated, detoxified L7-LOS to TT. This procedure was previously described for the synthesis of non-typeable *H. influenzae* LOS conjugates[30] in which internal Kdo residues were used as the linkage point. However, these residues are still close to, if not a part of, the inner epitopes. Therefore, consistent with our original strategy of stringently attempting to ensure the conservation of inner epitopes, we linked the O-deacylated LOS through its terminal reducing glucosamine residue. By distancing the linkage from the region of potential inner epitopes, we also hoped to avoid any possibility of linkage interference with this region. In order to conjugate L7-OH to TT by reductive amination, we needed to remove all the phosphate substituents from the hemiacetal group at C-1 of the lipid A terminal glucosamine residue. This was achieved by further treatment of the detoxified L7-LOS with alkaline phophatase. Using a combination of hydrazide and alkaline phosphatase treatments and following the procedures by ^{31}P NMR spectroscopy, we were able to substantiate the complete removal of both diphosphorylethanolamine groups from C-1 and C-4′ of the lipid A moiety. Although two monophosphate esters remained, one located at C-4′, which probably could have been removed by a more judicious enzyme treatment, the hemiacetal at C-1, critical to our conjugation procedure, was found to be completely free.

Therefore the above oligosaccharide (L7-OH, de P) and L7-OS were conjugated to TT by direct reductive amination, and both conjugates L7-OH, de P-TT and L7-OS-TT were shown by analysis to have similar saccharide/TT ratios. This together with their structural similarities, enabled a legitimate comparison of their immune performances to be made. Both conjugates were able to induce antibodies that bound to the L7-LOS, although the conjugate containing the conserved epitopes (L7-OH, de P-TT) was clearly the superior immunogen. That the conservation of inner L7-LOS epitopes is a factor in obtaining an efficacious immune response was more dramatically demonstrated in comparing the bactericidal activities of the antibodies induced by both conjugates. The conjugate having conserved inner saccharide epitopes (L7-OH, de P) induced antibodies that were more bactericidal against homologous organisms than antisera induced by the conjugate made with the truncated core oligosaccharide (L7-OS-TT). The poor bactericidal activity of the latter conjugate is consistent with previous studies using conjugates made with similar oligosaccharides of the L3 and L7 immunotypes.[12,20]

The difference in the ability of the two conjugates to induce bactericidal antibodies is surprising, because both conjugates were able to produce L7-LOS-binding

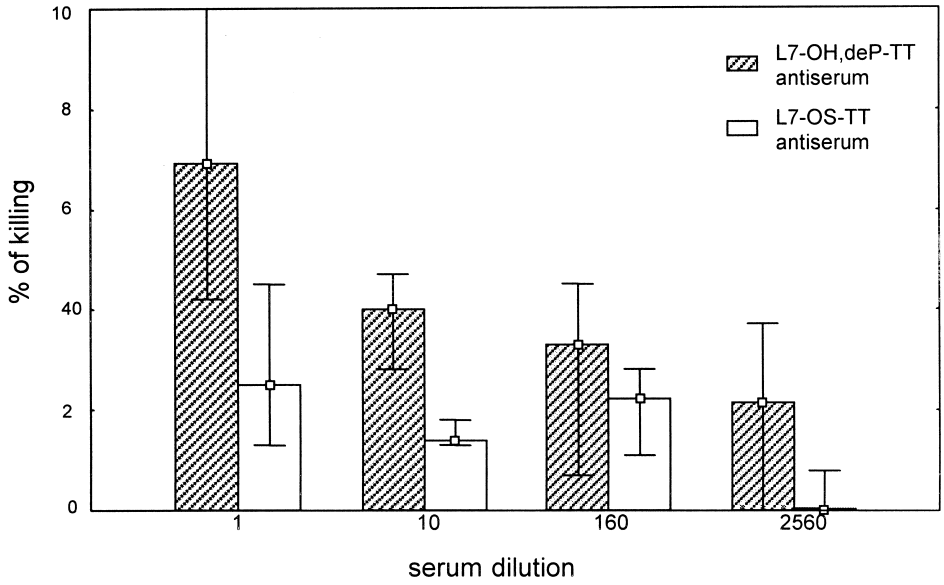

Fig. 3. Bactericidal activity of the pooled antisera of the ten mice immunized with either L7-OS-TT or L7-OH, de P-TT shown in Fig. 2. The bar graph represents the results of four different experiments. No bactericidal activity was observed in experiments carried out with complement but without antibody.

antibodies with isotypes, which at least had the potential of being bactericidal. Perhaps it is possible that the lack of bactericidal activity in the antibodies raised by L7-OS-TT could be explained by their low avidity, but our evidence is consistent with the fact that bactericidal epitopes are lost when the L7-LOS is subjected to mild acid hydrolysis.

4. Experimental

Strain M982B (serotype L7) was grown in Bacto Todd–Hewitt Broth (THB; Difco, Detroit, MI) at pH 7.3. Ten 5% chocolate agar plates (Quelab, Montreal, P.Q., Canada), were inoculated with bacteria from frozen stock and incubated overnight at 37 °C in an atmosphere of 5% CO_2. The bacteria were then resuspended in 50 mL of media (THB) and transferred to a screw-capped Erlenmeyer flask containing 2 L of media (THB). The flask was shaken for 7 h at 37 °C, and the contents were transferred to a 25-L New Brunswick Scientific MFS-128S Microferm fermentor. The bacteria were grown, killed with 1% formaldehyde, and harvested by centrifugation as previously described.

4.1. Isolation of LOS

LOS from the L7 serotype was isolated by a previously described modified phenol–extraction procedure.[14] It was finally purified by a fourfold ultracentrifugation for 6 h at $100{,}000 \times g$ using a Beckman LE-80 ultracentrifuge.

4.2. Analytical methods

Solutions were evaporated at reduced pressure below 40 °C in a rotary evaporator. Gel filtration was carried out on columns (1.6×90 cm) of Bio-Gel P2 and P4 (Extra Fine, Bio-Rad Laboratories), using 0.02 M pyridine–acetate buffer (pH 5.4) as eluant and a flow rate of 12 mL/h. A Sephadex G-10 column (1.5×30 cm, Pharmacia) was also employed, using water as the eluant at a flow rate of 24 mL/h. Individual fractions were monitored using a Waters R403 differential refractometer.

Conjugates were analysed for their carbohydrate and protein contents using respective phenol–sulfuric acid[31] and bicinchoninic acid assays.[32]

4.3. Nuclear magnetic resonance

NMR experiments were performed on a Bruker AMX, 500 spectrometer using a 5-mm broad band probe with ^{1}H coil nearest the sample. ^{1}H and ^{31}P NMR spectra were recorded at 300 and 340 K in 5-mm tubes at concentrations of 3–5 μg of sample in 0.5 mL of D_2O at pH 7.0 (^{1}H) and pH 7.6 (^{31}P). Acetone was used as the internal standard (2.225 ppm) for ^{1}H spectra. ^{31}P NMR samples contained 5 mM EDTA and 2% DOC and were referenced to 85% orthophosphoric acid (0.00 ppm). All experiments were carried out with sample spinning. 2D hetero-correlated (HSQC) experiments were performed as previously described.[33]

4.4. Chemical methods

O-Deacylation of the L7-LOS was performed using anhydrous hydrazine as previously described,[22] to yield O-deacylated and partially dephosphorylated L7-OH. The core oligosaccharide (L7-OS) was obtained by heating the LOS (10 mg/mL) in 1% HOAc at 100 °C for 2 h. The insoluble lipid A was removed by centrifugation at 15,000 rpm for 15 min, and the water-soluble L7-OS was purified on a Bio-Gel P2 column.

4.5. Enzymatic dephosphorylation of LOS-OH

LOS-OH (10 mg) was dissolved in 1 mL of 0.1 M ammonium bicarbonate (pH 8.0) and treated with 70 units of alkaline phosphatase (Boehringer Mannheim, Laval, P.Q., Canada) at 56 °C for 18 h. At this time an additional 70 units of the same enzyme were added, and the reaction was allowed to stand at 56 °C for a further 6 h. The solution was then heated at 100 °C for 5 min, centrifuged at 15,000 rpm for 5 min, and the partially dephosphorylated product (LOS-OH, de P) was purified on a Sephadex G-10 column.

4.6. Coupling of L7-OH, de P to tetanus toxoid

Using reductive amination procedures previously described,[5,12] L7-OH, de P was conjugated to TT. L7-OH, de P (10 mg) was dissolved in 200 μL of 0.02 M borate buffer at pH 9.0, together with TT (4 mg) and sodium cyanoborohydride (50 mg). The reaction was stirred for 4 d at 37 °C, and the progress of the conjugation was monitored by a Hewlett–Packard Model 1100 HPLC using a Superose 12 HR10/30 column (Pharmacia) with PBS as eluant at a flow rate of 0.4 mL/min. The eluant was monitored using a UV detector operating at OD_{214} and OD_{280}. Conjugation was indicated by the gradual disappearance of the TT peak with the simultaneous appearance of the conjugate peak having a relatively lower K_{av} value. When the TT peak had disappeared, the conjugate was purified on a Bio-Gel A 0.5 (Bio-Rad) column (1.6×42 cm) as was obtained in a yield of 5 mg. L7-OS was conjugated to TT using identical procedures.[12]

4.7. Immunization procedures

Groups of 6–8 week old 10 CF1 female mice (Charles River, St. Constant, Canada) were injected subcutaneously with each of the conjugates containing 2.5 μg of carbohydrate in saline solution, together with RIBIs complete adjuvant (RIBI Immunochem Research, Inc., Hamilton, MT) in a total volume of 0.2 mL. The mice were injected on day 0, 21, and 35, and the antisera were collected on day 45, filtered sterile and stored at −80 °C.

4.8. ELISA

The wells of microtiter plates (Lynbro/Titertek, No. 76-381-04) were coated with solutions of LOS (2 μg/100 μL) in 0.05 M sodium carbohydrate buffer at pH 9.6 at 37 °C for 3 h and then overnight at 4 °C. The plates were then washed and blocked with 1% BSA in 20 mM Tris-HCl–50 mM NaCl buffer containing 0.05% Tween 20 (T-TBS) pH 7.5 for 1 h at room temperature. The contents of the wells were then removed, serial dilutions (100 μL/well) of murine antisera in PBS were added, and the plates were left for 2.5 h at room temperature. After washing with T-TBS, 100 μL of a 1:3000 dilution in PBS of an alkaline phosphatase-labelled goat anti-mouse IgG (H + L) (ICN, Aurora, OH) was added to each well. Following incubation for 1 h at room temperature, the plates were washed with T-TBS (250 μL/well), and 100 μL/well of pNPP substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added. The plates were allowed to stand for 1 h at room temperature, and the optical densities were read at 410 nm using a Dynatech MR 5000 Microplate Reader. Isotyping experiments were performed using the same ELISA procedure as described above except with the substitution of peroxidase-labeled goat anti-mouse isotype-specific antibodies (Southern Biotechnology Associates, Birmingham, AL) and the reading of the plates at 450 nm.

4.9. Bactericidal assay

The bactericidal assays were carried out in tissue culture 96-well polystyrene plates (Costar, No. 3595) essentially as previously described.[34] *N. meningitidis* strain M982B (GBM) was grown overnight on chocolate agar plates (Quelab, Montreal, P.Q. Canada) at 37 °C under a 5% CO_2 atmosphere, followed by inoculating a second plate and incubating it for 5 h. Two-fold dilutions of murine polyclonal antisera were made directly in the plate using HBSS containing 1% casein hydrolyzate, diluted to a final volume of 50 μL/well. A suspension of GBM in HBSs, 1% casein hydrolyzate was made giving an $OD_{490} = 0.29$ and a final working dilution of bacteria was prepared by a further 1:20,000 dilution. Freshly thawed baby rabbit complement was added (20 μL) to each well, followed by 30 μL of the working dilution of bacteria (2,500 CFU/well). The plate was then shaken at 37 °C for 1 h. The content of each well was then mixed before plating (10 μL) onto chocolate agar. The agar plates were incubated overnight at 37 °C, 5% CO_2, and the number of CFU were counted. The percent of killing was calculated relative to the mean values of either HBSS control wells or culture supernatant medium in the following manner: percentage of killing $= (CFU_{control} - CFU_{mAb}/CFU_{control}) \times 100$.

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