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Structure, Serological Specificity, and Synthesis of Artificial Glycoconjugates Representing the Genus-Specific Lipopolysaccharide Epitope of *Chlamydia* spp.

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The human bacterial pathogens *Chlamydia* spp. possess a genus-specific lipopolysaccharide as a major surface antigen, the structure of which has been determined by analytical chemistry as Kdoα2-8-Kdoα2-4-Kdoα2-6GlcNpβ1-6GlcNol (Kdo, 3-deoxy-D-*manno*-2-octulosonic acid). Immunochemical studies on this pentasaccharide and the chemically synthesized partial structures Kdoα2-8-Kdoα2-4-Kdoα2-6GlcNpβ, Kdoα2-8-Kdoα2-4-Kdoα, Kdoα2-4-Kdoα, Kdoα2-8-Kdoα, and Kdoα using artificial glycoconjugate antigens and monoclonal antibodies showed that fatty acids and phosphoryl groups (as present in native lipopolysaccharide) are dispensable for constitution of the genus-specific epitope and that the minimal structure to exhibit chlamydia specificity is the Kdo trisaccharide moiety.

The genus *Chlamydia*, with the species *Chlamydia psittaci*, *C. trachomatis*, and *C. pneumoniae*, represents pathogenic, obligatory intracellular parasites causing a variety of acute and chronic diseases in animals and humans. More than 500 million people in the third world suffer from trachoma, the world's leading cause of secondary blindness. In developed countries, *C. trachomatis* is the major cause of sexually transmitted genital infections (2, 25).

Little is known about the pathogenicity factors of these unique microorganisms and the host defense mechanisms against them, but undoubtedly, surface structures of chlamydiae are involved in the early steps of infection (adhesion and penetration). At the same time, they are surface antigens against which antibodies are made during infection.

The major surface antigens of chlamydiae are a 39.5-kDa major outer membrane .protein and lipopolysaccharide (LPS). The latter is chemically and serologically related to the LPS of Re mutants of enterobacteria (6, 8–10, 21–23), which is composed of an α2.4-linked disaccharide .of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) and lipid A, a phosphorylated and acylated D-glucosamine disaccharide (4). In addition, chlamydial LPS harbors a genus-specific epitope which has not been detected in any other bacterium, including enterobacterial Re mutants (10). The tools of molecular genetics have enabled cloning (20) and expression of the enzyme(s) involved in the biosynthesis of the chlamydia-specific epitope in Re mutant *Salmonella minnesota* R595 (7). Chemical investigations of such recombinant and chlamydial LPS have shown that they contain a Kdo trisaccharide with the sequence Kdo2-8Kdo2-4Kdo, which differs from the LPS of the parent strain by the presence of a third Kdo residue linked to position 8 of the terminal Kdo of the 2.4-linked disaccharide (3). However, the anomeric configurations of the Kdo residues and the linkage between Kdo and the glucosamine of the lipid A moiety could not be determined in a direct way. In addition, although the third Kdo is a prerequisite for the genus-specific epitope, the minimal structure that expresses chlamydia specificity is not known. To answer the open questions as to the chemical and antigenic structures of chlamydial and recombinant LPS, we prepared chemically homogeneous structures that exhibit chlamydia specificity and are suitable for analysis by combined gas-liquid chromatography–mass spectrometry (GLC-MS) and nuclear magnetic resonance (NMR) spectroscopy. The results obtained are described herein.

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MATERIALS AND METHODS

Recombinant LPS. Re mutant of *S. minnesota* R595 was transformed (7) with plasmid pFEN207 (20). LPS was extracted from the resulting recombinant, purified, and converted to the uniform triethylammonium salt after electrodialysis as previously reported (12, 13).

Isolation and purification of the pentasaccharide. De-*O*-acylated and dephosphorylated LPS was prepared in the following way. LPS (100 mg) was dried overnight in a desiccator over phosphorus pentoxide, dissolved in anhydrous hydrazine (5 ml), and stirred for 30 min at 37°C. After addition of chilled (−20°C) acetone, the precipitate was collected by centrifugation, washed extensively with acetone, dissolved in water (5 ml), reprecipitated with acetone, dried, dissolved in water (5 ml), and lyophilized, leading to formation of de-*O*-acylated LPS. The dry sample was suspended in 48% aqueous hydrogen fluoride (5 ml) in a polypropylene tube and stirred at 4°C for 2 days. After centrifugation, the sediment was washed several times with ethanol, dissolved in water (5 ml), and lyophilized; the yield was 40 to 60% based on LPS. De-*O*-acylated and dephosphorylated LPS (20 mg) was dissolved in water (2 ml), followed by addition of sodium borohydride (20 mg) and magnetic stirring at room temperature for 1 h. The reaction mixture was acidified with acetic acid and evaporated several times to dryness after addition of methanol, followed by

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dialysis against water and lyophilization, yielding de-*O*-acylated, dephosphorylated, and reduced LPS, which was treated with anhydrous hydrazine at 85°C for 7 days to remove the remaining amide-linked fatty acids. Hydrazine was removed under vacuum in a desiccator over sulfuric acid. A solution of the residue in water (5 ml) was passed through a column (2.5 by 5 cm) filled with polyethylenimine cellulose (medium mesh, 0.94 meq/g), washed with water (50 ml), and eluted with pyridinium acetate (50 ml, 50 mM, pH 5.5). The eluate was lyophilized, and a solution of the residue in water (0.5 ml) was subjected to preparative high-voltage paper electrophoresis (40 V/cm; pyridine-acetic acid-formic acid-water [1:10:1.5:90, pH 2.8]), followed by staining with alkaline silver nitrate. The fraction with M = 0.21 relative to Kdo (M = 1.0) was eluted and lyophilized. Thus, a total of 25 mg of the amorphous pentasaccharide was obtained.

GLC-MS and ^{13}C-NMR spectroscopy. The pentasaccharide was dissolved in D_2O (0.5 ml), brought to pH 8.0 with NaOD, and subjected to ^{13}C-NMR spectroscopy at room temperature using a Bruker AM360L instrument at 90.56 MHz in the Fourier transform mode with complete proton decoupling. Chemical shifts are given in δ units (parts per million downfield from an external signal of acetonitrile [δ 1.70 ppm]). GLC-MS was performed as previously reported (3).

Serology. Monoclonal antibody S5-10 (immunoglobulin G3), which recognizes a genus-specific epitope of chlamydial LPS, was used for inhibition experiments in the passive hemolysis inhibition assay (6). Briefly, a constant amount of antibody was preincubated with serial dilutions of inhibitor for 15 min at 37°C, followed by incubation with sheep erythrocytes coated with recombinant r595-207 LPS and guinea pig serum as a source of complement. Inhibitor dilutions causing 50% inhibition were determined, and inhibition values were calculated as molar concentrations.

Synthetic Kdo oligosaccharides. The allyl glycosides sodium (allyl 3-deoxy-α-D-*manno*-2-octulopyranosyl)onate, disodium [3-deoxy-α-D-*manno*-2-octulopyranosylonate-(2-4)-(allyl 3-deoxy-α-D-*manno*-2-octulopyranosyl)]onate, disodium [3-deoxy-α-D-*manno*-2-octulopyranosylonate-(2-8)-(allyl 3-deoxy-α-D-*manno*-2-octulopyranosyl)]onate, trisodium [3-deoxy-α-D-*manno*-2-octulopyranosylonate-(2-8)-3-deoxy-α-D-*manno*-2-octulopyranosylonate-(2-4)-(allyl 3-deoxy-α-D-*manno*-2-octulopyranosyl)]onate, and trisodium [3-deoxy-α-D-*manno*-2-octulopyranosylonate-(2-8)-3-deoxy-α-D-*manno*-2-octulopyranosylonate-(2-4)-(3-deoxy-α-D-*manno*-2-octulopyranosyl)]onate-(2-6)-allyl-2-acetamido-2-deoxy-β-D-glucopyranoside were synthesized as previously reported (15–17). The glycoside moieties of these compounds will be abbreviated as Kdo, 2.4-Kdo_2, 2.8-Kdo_2, Kdo_3, and Kdo_3-GlcNAc, respectively.

Synthesis of cysteamine glycosides. Reactions of the allyl glycosides of Kdo, 2.4-Kdo_2, 2.8-Kdo_2, Kdo_3, and Kdo_3-GlcNAc with 3-(2-aminoethylthio)propyl (cysteamine) were performed as previously reported (19). The products were isolated in 40 to 72% yields by ion-exchange chromatography on Dowex AG50WX-4 (NH_4^+ form) by using 0.1 M aqueous NH_3 as the eluant and desalted on Bio-Gel P-2. ^{1}H-NMR spectra containing triplets at δ 3.20 ppm (N-CH_2), 2.60 to 2.70 and 2.80 to 2.88 ppm (-CH_2-S-CH_2), 1.80 ppm (doublet of triplets) (-CH_2) and ^{13}C-NMR signals at 39.0 to 40.0 ppm (N-CH_2), 29.1 to 29.8 ppm (-CH_2-S-CH_2), and 28.2 to 28.7 ppm (-CH_2) were in agreement with the structures assigned.

Synthesis of artificial glycoconjugate antigens. (i) Allyl glycosides were copolymerized with acrylamide as previously described (14), yielding polyacrylamide derivatives (Fig. 1, compound II). (ii) The spacer arm glycosides of Kdo_3 and Kdo_3-GlcNAc were reacted with acryloyl chloride (24) in oxolane at 0°C with maintenance of the pH at 7.5 by continuous addition of 0.1 M aqueous NaOH to give a quantitative yield of the corresponding acrylamide derivatives (Fig. 1, compound V). ^{1}H-NMR spectra revealing signals at δ 6.20, 6.08, and 5.66 ppm and ^{13}C-NMR signals at 130.8 and 128.3 ppm proved the presence of the acrylamide residue (data not shown). After copolymerization with acrylamide, these compounds yielded derivatives in which the ligand was separated by approximately 1 nm from the polyacrylamide chain. (iii) The spacer arm glycosides of Kdo, 2.4-Kdo_2, 2.8-Kdo_2, Kdo_3, Kdo_3-GlcNAc (Fig. 1, compound III), and the pentasaccharide (via its free amino groups of the glucosamine and glucosaminitol residue) were also covalently linked to bovine serum albumin (BSA) by the glutardialdehyde method as follows. BSA (10 mg) was preactivated with glutardialdehyde (final concentration, 5%)

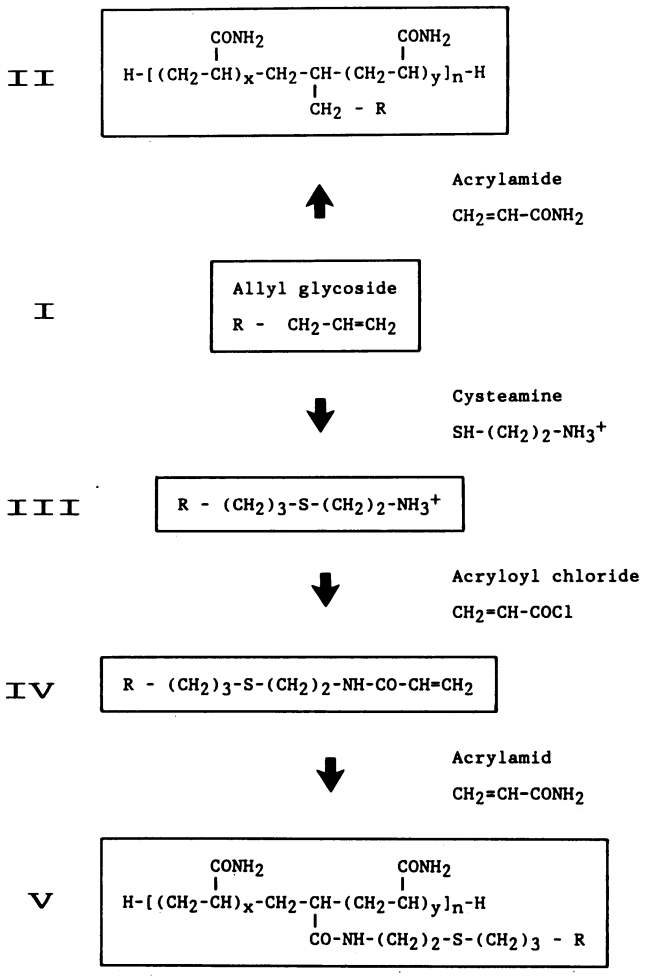

FIG. 1. General derivatization pathway of Kdo oligosaccharides for preparation of synthetic glycoconjugates starting from the allyl glycosides (I). Copolymerization of compound I or IV with acrylamide resulted in formation of compounds II and V, respectively. The cysteamine-spacered compound can be coupled to protein or other carriers via its primary amino group. R represents Kdo, 2.4-Kdo_2, 2.8-Kdo_2, Kdo_3, or Kdo_3-GlcNAc.

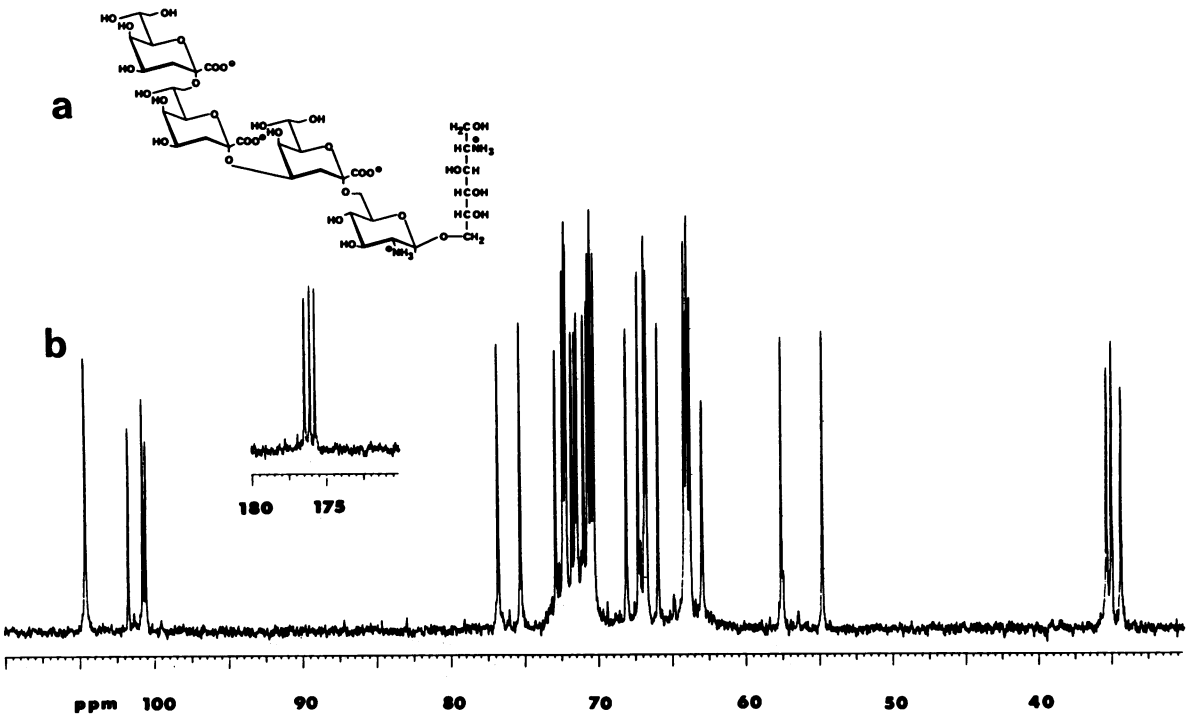

FIG. 2. Chemical structure of the pentasaccharide carbohydrate backbone Kdopα2-8Kdopα2-4Kdopα2-6GlcNpβ1-6GlcNol of recombinant LPS (a) and its ^{13}C-NMR spectrum (b).

under nitrogen at room temperature in 50 mM carbonate buffer, pH 9.2 (250 μl), followed by dialysis against the same buffer. Cysteamine-spacered ligands (2 mg) were added to the retentate, and formation of the Schiff bases was allowed at room temperature overnight, followed by reduction with sodium borohydride and dialysis against phosphate-buffered saline. The products were sterilized by filtration (0.22 μm) and stored at 4°C. The ligand amounts present in the synthetic glycoconjugates were determined by measuring Kdo contents by the thiobarbiturate assay after hydrolysis in acetate buffer as previously described (5).

RESULTS AND DISCUSSION

The recombinant LPS was composed of two major LPS populations containing, apart from lipid A, two and three Kdo residues, respectively. Previous experiments have shown that dephosphorylated and de-*O*-acylated LPS still expressed the chlamydia-specific epitope (6). These results suggested that the immunodominant group was located in the carbohydrate moiety. Therefore, we developed a degradation protocol allowing preparation of large-scale quantities of the carbohydrate backbone of recombinant LPS.

Isolation and purification of the pentasaccharide carbohydrate backbone Kdo-Kdo-Kdo-GlcN-GlcNol of recombinant LPS. The protocol for isolation and purification of the pentasaccharide carbohydrate backbone included (i) removal of ester-linked fatty acids of the lipid A moiety by mild hydrazinolysis, (ii) cleavage of phosphate groups with hydrofluoric acid without affecting the ketosidic bonds of Kdo, (iii) reduction of the reducing glucosamine residue of lipid A with sodium borohydride, and (iv) cleavage of the amide-linked fatty acids in lipid A with hydrazine at high temperature. The reaction mixture obtained contained a pentasaccharide consisting of one glucosaminitol, one glucosamine, and three Kdo residues, in addition to several degradation products. Basic components were separated from acidic ones by ion-exchange chromatography whereby the latter were found in the fraction eluting with pyridinium acetate. Further purification by preparative high-voltage paper electrophoresis separated the pentasaccharide from other contaminants. Thus, a total of 25 mg of the homogeneous pentasaccharide was prepared.

Structural analysis of the pentasaccharide carbohydrate backbone Kdo-Kdo-Kdo-GlcN-GlcNol of recombinant LPS. Figure 2a shows the chemical structure of the pentasaccharide carbohydrate backbone of recombinant LPS as determined by ^{1}H- and ^{13}C-NMR spectroscopy and combined GLC-MS. The ^{13}C-NMR spectrum is shown in Fig. 2b. It displayed signals for all 36 carbon atoms, which were partially assigned by empirical correlation (Table 1) with synthetic model compounds such as Kdo (1), 2.4-Kdo_2 (11), 2.8-Kdo_2 (17), Kdo_3 (17), and Kdo_3-GlcNAc (15). The characteristic signals for the three Kdo residues were those at 175.71, 176.03, and 176.40 ppm for C-1 (carboxyl carbons), those at 34.39, 35.02, and 35.35 ppm for C-3 (deoxy group), and those for the glycosidic carbons C-2 at 101.70, 100.55, and 100.75 ppm. The α-anomeric configuration of the Kdo residues was inferred from ^{13}C-NMR chemical shift values of the C-1 signals and the ^{1}H-NMR chemical shift differences between the equatorial and axial protons at C-3, which were observed at δ 2.05 to 1.64 ppm (1). Substitution of one Kdo at C-4 was indicated by glycosylation shifts of the corresponding and neighboring signals at 70.26, 34.39, and 65.94 ppm, respectively (11). The three signals for C-8 at 63.93, 64.01, and 64.19 ppm could not be assigned unambiguously but compared favorably to similar values for a synthetic Kdo trisaccharide (17). The signals for a 6-*O*-substituted β-pyranosidic glucosamine residue and a 6-*O*-substituted glu-

TABLE 1. Empirical assignment of ^{13}C-NMR chemical shifts of the pentasaccharide, the tetrasaccharide, and the trisaccharide

Sugar moiety[a] and carbon atom	Chemical shift value (ppm) obtained for:[b]		
	A	B	C
GlcNol			
1	62.96		
2	54.77		
3	71.40 (i)		
4	71.53 (i)		
5	71.78 (i)		
6	72.88		
6-*O*-GlcN			
1	104.57	101.35	101.10
2	57.57	56.41	56.39
3	75.28	74.82	75.05 (i)
4	70.96 (ii)	70.41 (i)	70.41 (ii)
5	76.79	75.11	75.10 (i)
6	63.67	63.21	62.40
4-*O*-Kdo*p*			
1	175.71 (iii)	175.77	175.74
2	101.70 (iv)	101.00	100.69
3	34.39	34.30	34.09
4	70.26	71.73 (ii)	69.21
5	65.94	65.60	65.01
6	72.21 (v)	72.13 (iii)	72.35
7	70.62 (ii)	71.36 (ii)	71.08
8	64.03 (vi)	64.03 (iv)	64.25 (iii)
8-*O*-Kdo*p*			
1	176.40 (iii)	176.36 (v)	
2	100.55 (iv)	100.56 (vi)	
3	35.02	35.04	
4	66.70 (vii)	66.69 (vii)	
5	68.08	67.96 (viii)	
6	72.36(v)	72.62	
7	70.70(ii)	70.47 (i)	
8	64.19 (vi)	64.15 (iv)	
Terminal Kdo*p*			
1	176.03 (iii)	175.99 (v)	176.50
2	100.75 (iv)	100.69 (vi)	100.09
3	35.35	35.34	35.33
4	66.81 (vii)	66.79 (vii)	66.77
5	67.26	67.24 (viii)	66.99
6	72.15 (v)	72.03 (iii)	73.30
7	70.46 (ii)	70.14 (i)	70.54 (ii)
8	63.93 (vi)	63.88 (iv)	64.21 (iii)

[a] GlcNol, D-Glucosaminitol; GlcN, D-glucosamine (in compounds B and C, this carbohydrate residue is *N*-acetyl-D-glucosamine); Kdo*p*, 3-deoxy-D-*manno*-octulopyranosonic acid.

[b] A, Kdo$p\alpha$2-8Kdo$p\alpha$2-4Kdo$p\alpha$2-6GlcN$p\beta$1-6GlcNol; B, Kdo$p\alpha$2-8Kdo$p\alpha$2-4Kdo$p\alpha$2-6GlcNAc$p\beta$1-allyl; C, Kdo$p\alpha$2-4Kdo$p\alpha$2-6GlcNAc$p\beta$1-allyl. Similarly labeled assignments within a column are interchangeable. The data for B and C are from reference 17.

cosaminitol were in accordance with those previously published (18). From these data, it was evident that the pentasaccharide was composed of a reducing glucosaminitol residue substituted in position 6 by a β-pyranosidic glucosamine which, in turn, carries a Kdo trisaccharide at its 6-hydroxyl group. The NMR data also showed that all three Kdo residues are α-pyranosides and that one Kdo is substituted at position 4. Further information on the linkages between the Kdo residues was obtained by combined GLC-MS. The pentasaccharide was hydrolyzed, carbonyl reduced, and permethylated to give a mixture of Kdo mono- and oligosaccharides which was separated by gas-liquid chromatography. Chemical ionization mass spectrometry identified a Kdo trisaccharide, two disaccharides, and a Kdo monosaccharide. Electron impact mass spectrometry showed that the disaccharides were 2.4 and 2.8 linked and that the trisaccharide was a linear one with a 4-*O*-substituted Kdo at the reducing end (data not shown; see references 3 and 17). These data, together with the NMR spectroscopy data, are in accordance with the structural formula given in Fig. 2a.

TABLE 2. Inhibition of chlamydia-specific monoclonal antibody S5-10 by synthetic glycoconjugates of Kdo oligosaccharides

Inhibitor	Ligand concn (nmol/mg)	Inhibitory concn (μM)
Copolymerization products		
Kdo-PA[a]	323	>10
2.4-Kdo_2–PA	338	>10
2.8-Kdo_2–PA	295	>10
Kdo_3–PA	106	5.30
Kdo_3–PA (spacered)	275	0.03
Kdo_3–GlcNAc-PA (spacered)	140	0.04
BSA conjugates		
Kdo-BSA	64	>10
2.4-Kdo_2–BSA	9	>10
2.8-Kdo_2–BSA	19	>10
Kdo_3-BSA	17	0.34
Kdo_3-GlcNAc-BSA	17	0.34
Pentasaccharide-BSA	16	0.32

[a] PA, Polyacrylamide.

Synthesis of artificial glycoconjugates. On the basis of the chemical structure established for the pentasaccharide, the partial structures Kdo, 2.4-Kdo_2, 2.8-Kdo_2, Kdo_3, and Kdo_3-GlcNAc were synthesized and used to prepare artificial glycoconjugate antigens suitable for study by serological methods. Various derivatization protocols for carbohydrates that allow their immobilization or coupling to proteins have been described. We chose as a first target for all ligands their allyl glycosides, since they could be copolymerized with acrylamide to yield inert, high-molecular-weight antigens. On the other hand, condensation of the allyl glycosides with cysteamine yielded spacered glycosides with a primary amino group by which the ligand could be immobilized or covalently linked to BSA. In addition, cysteamine-spacered Kdo_3 and Kdo_3-GlcNAc were reacted with acryloyl chloride, which also resulted in introduction of a terminal double bond by which the compounds could be copolymerized with acrylamide. This type of copolymerization product differs from those obtained from the allyl glycosides in that the ligand is separated from the polyacrylamide chain by a spacer. The general reaction sequence and formulas of the products are shown in Fig. 1, and the synthetic glycoconjugates prepared in this study are listed in Table 2; which also displays the concentrations of ligand incorporated.

Serological analysis. The immunoreactive properties of the glycoconjugates were determined in a passive hemolysis inhibition assay using a chlamydia-specific monoclonal antibody (Table 2). The Kdo monosaccharide and the two disaccharides were inactive (>10 μM), independently of whether copolymers or BSA conjugates were used. The Kdo trisaccharide was most active as a copolymer containing the spacered ligand Kdo_3-$(CH_2)_3$-S-$(CH_2)_2$-NH-CO-CH=CH_2 and yielded an inhibition value similar to that of the tetrasac-

charide Kdo_3-GlcNAc. This indicated that the Kdo trisaccharide is the minimal structure for expression of chlamydia specificity in terms of binding to monoclonal antibody S5-10. This was confirmed by the similar inhibition values obtained with the BSA conjugates of Kdo_3, Kdo_3-GlcNAc, and the pentasaccharide. In addition, the results suggested that the tertiary structures of the conjugates or the conformations of the carbohydrate ligands were different in unspacered and spacered copolymerization antigens. Obviously, free access of the antibody to its epitope is achieved only by local separation of the ligand from the polyacrylamide chain by a spacer.

In summary, we have elucidated the chemical structure of the carbohydrate backbone of a recombinant LPS which contains a genus-specific epitope of the human pathogens *Chlamydia* spp., and we have synthesized immunoreactive glycoconjugates with biological activities identical to those of their natural counterparts. These reagents are helpful in studying the interaction of chlamydiae with host cells on the molecular level, and they may prove useful for development of vaccines and new diagnostic reagents.

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