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A Synthetic Glycoconjugate Representing the Genus-Specific Epitope of Chlamydial Lipopolysaccharide Exhibits the Same Specificity as Its Natural Counterpart

YUCAI FU,[1] MARITTA BAUMANN,[1] PAUL KOSMA,[2] LORE BRADE,[1] AND HELMUT BRADE[1]*

Division of Biochemical Microbiology, Institut für Experimentelle Biologie und Medizin, Forschungsinstitut Borstel, Parkallee 22, D-2061 Borstel, Germany,[1] and Institut für Chemie der Universität für Bodenkultur, A-1180 Vienna, Austria[2]

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The tetrasaccharide 3-deoxy-α-D-*manno*-2-octulosonic acid (α-KDO)(2→8)-α-KDO(2→4)-α-KDO(2→6)-βGlcNAc, a partial structure of chlamydial lipopolysaccharide (LPS) representing a genus-specific epitope, was synthesized and covalently linked to bovine serum albumin, resulting in an artificial glycoconjugate antigen. Mice were immunized with the glycoconjugate to prepare chlamydia-specific monoclonal antibodies. They were selected with chlamydia-specific LPS antigens and the structurally and antigenically related Re-type LPS of a *Salmonella minnesota* rough mutant. Characterization of the selected antibodies was by (i) hemagglutination of sheep erythrocytes coated with recombinant chlamydia-specific LPS, (ii) inhibition by synthetic polyacrylamide derivatives containing the genus-specific epitope or partial structures thereof, (iii) enzyme immunoassay with recombinant LPS and synthetic bovine serum albumin glycoconjugates as solid-phase antigens, (iv) immunofluorescence of L929 monolayers infected with *Chlamydia psittaci* or *C. trachomatis*, and (v) Western immunoblots with glycoconjugates and LPS as the antigen. Two groups of monoclonal antibodies were obtained; the monoclonal antibodies in one group cross-reacted with chlamydial and Re-type LPS, but those of the other group were chlamydia specific. Among the latter, KDO trisaccharide-specific antibodies that had the same epitope specificity as antibodies obtained after immunization with chlamydial elementary bodies were identified; however, they exhibited a more than 100-fold higher affinity. In addition, antibodies that bound preferentially to the 2.8-linked KDO disaccharide were detected, although with lower affinity. The data show that the artificial glycoconjugate antigen is similar to its natural counterpart.

Bacteria of the genus *Chlamydia* are pathogenic, obligatory phagosomal intracellular parasites that cause acute and chronic diseases in animals and humans. *Chlamydia psittaci*, *C. trachomatis*, and *C. pneumoniae*, which are species of the monogeneric family *Chlamydiaceae*, have a developmental cycle in which infectious, metabolically inactive elementary bodies and noninfectious, multiplying reticulate bodies are produced (21, 29). The pathogenic potential of these microorganisms is not known. However, recent data on the surface antigens of chlamydiae, in particular on the 39.5-kDa major outer membrane protein and the lipopolysaccharide (LPS), lead one to speculate on their significance as virulence factors. The antigenic activity of the LPS is shared by all chlamydiae, and thus the LPS represents a genus-specific antigen (10). Elucidation of the molecular structure of chlamydial LPS was hampered by poor growth of the microorganism in quantities large enough to allow purification and structural analysis of isolated LPS. The tools of molecular biology are hardly adaptable to chlamydial genetics, since no controlled system of recombination is available. The cloning and expression of chlamydial genes in *Escherichia coli* (31) and production of monoclonal antibodies to chlamydial LPS (9) allowed the cloning of a DNA fragment of chlamydial genomic DNA expressing the genus-specific antigen (22). The molecular basis of this phenomenon was studied by transforming enterobacterial Re mutants (3, 6) with the cloned chlamydial gene(s) and comparing the structures of the recombinant and parent LPSs. Studies such as these showed that the chlamydia-specific LPS contained, in its saccharide portion, a trisaccharide of 3-deoxy-D-*manno*-2-octulosonic acid (KDO) of the sequence α-KDO (2→8)-α-KDO(2→4)-α-KDO (KDO_3) (1). The 2.4-linked disaccharide part of the trisaccharide was from the parent Re LPS, and the 2.8-linked moiety is the structural characteristic of chlamydial LPS. At the same time, the appearance of the 2.8-linked third KDO was paralleled by the concomitant expression of the genus-specific chlamydial epitope (1). Thus, the KDO region represented the immunodominant segment of chlamydial LPS. The complete carbohydrate backbone of chlamydia-specific recombinant LPS, isolated after deacylation and dephosphorylation, is a pentasaccharide in which the KDO trisaccharide is α-2.6 linked to the C-6′ of the β1.6-linked glucosamine disaccharide of lipid A (13). These analytical data were confirmed by chemical synthesis, which also allowed the synthesis of artificial glycoconjugates (15, 17). These compounds were serologically characterized by using monoclonal antibodies obtained after immunization with whole elementary bodies. The minimal structure to which these antibodies bound was the KDO trisaccharide (13). In addition to the chlamydia-specific epitope, other epitopes were identified in the KDO region; i.e., the α-pyranosidically linked terminal KDO and the α-2.4-linked disaccharide portion (28). These latter structures in other LPSs (3) explain serological cross-reactions between chlamydiae and various gram-negative bacteria (5, 7–9, 23, 24).

Do these synthetic antigens also represent artificial immunogens when injected into experimental animals? We report on the immunization of mice with synthetic α-KDO(2→8)-

* Corresponding author.

TABLE 1. Chemical structures of carbohydrate ligands present in artificial synthetic glycoconjugates

Chemical structure of carbohydrate ligands	Nature of R	Abbreviation	Amt of ligand[a] (nmol/mg)
KDO-$(CH_2)_3$-S-$(CH_2)_2$-NH-R	PA[b]	Kdo-PA	662
KDO-(2→4)-KDO-$(CH_2)_3$-S-$(CH_2)_2$-NH-R	PA	2.4-KDO_2-PA	278
KDO-(2→8)-KDO-$(CH_2)_3$-S-$(CH_2)_2$-NH-R	PA	2.8-KDO_2-PA	320
KDO-(2→8)-KDO-(2→4)-KDO-$(CH_2)_3$-S-$(CH_2)_2$-NH-R	PA	KDO_3-PA	275
KDO-(2→8)-KDO-(2→4)-KDO-(2→6)-GlcNAc-$(CH_2)_3$-S-$(CH_2)_2$-NH-R	PA	KDO_3-GlcNAc-PA	140
KDO-$(CH_2)_3$-S-$(CH_2)_2$-NH-CS-NH-R	BSA	KDO-BSA	119
KDO-(2→4)-KDO-$(CH_2)_3$-S-$(CH_2)_2$-NH-CS-NH-R	BSA	2.4-KDO_2-BSA	44
KDO-(2→8)-KDO-$(CH_2)_3$-S-$(CH_2)_2$-NH-CS-NH-R	BSA	2.8-KDO_2-BSA	66
KDO-(2→8)-KDO-(2→4)-KDO-$(CH_2)_3$-S-$(CH_2)_2$-NH-CS-NH-R	BSA	KDO_3-BSA	38
KDO-(2→8)-KDO-(2→4)-KDO-(2→6)-GlcNAc-$(CH_2)_3$-S-$(CH_2)_2$-NH-CS-NH-R	BSA	KDO_3-GlcNAc-BSA	55

[a] Determined by the thiobarbiturate assay (2).
[b] PA, polyacrylamide.

α-KDO(2→4)-α-KDO(2→6)-GlcNAc (KDO_3-GlcNAc) covalently linked to a protein carrier. We show below that the antibodies selected have the same specificity but a significantly higher affinity than those prepared against the native chlamydial LPS.

MATERIALS AND METHODS

Bacteria and bacterial LPS. The rough mutant of *Salmonella minnesota* chemotype Re (strain R595) was transformed (6) with plasmid pFEN207 (22). LPSs from the resulting recombinant and the parent strain were extracted with phenol-chloroform-petroleum ether (12) and purified by repeated ultracentrifugation. Finally, the LPSs were converted to uniform triethylammonium salts after electrodialysis (11) and are called r595-207 and R595. Partially de-O-acylated and de-O-acylated and dephosphorylated LPSs were prepared as reported elsewhere (27). The formation of LPS-bovine serum albumin (BSA) complexes was carried out by mixing equal quantities in water (5 mg/ml) and then subjecting the samples to three cycles of lyophilization and solubilization.

Artificial antigens. (i) Chemical synthesis of carbohydrate ligands. 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 reported previously (15–17).

(ii) Synthesis of polyacrylamide derivatives containing carbohydrate ligands with spacers. The above allylglycosides were reacted with cysteamine as reported previously (20), leading to ligands with spacers of the general formula R-$(CH_2)_3$-S-$(CH_2)_2$-NH_3^+, where R represents the respective carbohydrate ligand (Table 1). These compounds were reacted with acryloyl chloride (26), resulting in the formation of derivatives of the general formula R-$(CH_2)_3$-S-$(CH_2)_2$-NH-CO-CH=CH_2, which, after copolymerization with acrylamide (14), yielded polyacrylamide derivatives of the general formula shown in Fig. 1A.

(iii) Synthesis of BSA-glycoconjugates containing spacered carbohydrate ligands with spacers. Ligands with spacers of the general formula R-$(CH_2)_3$-S-$(CH_2)_2$-NH_3^+ were activated into isothiocyanate derivatives as follows. Thiophosgen (6 μl) was dissolved in chloroform (3 ml), and a solution of the ligand with spacers (15 mg) in water (3 ml) was added. The reaction was monitored by using thin-layer chromatog-

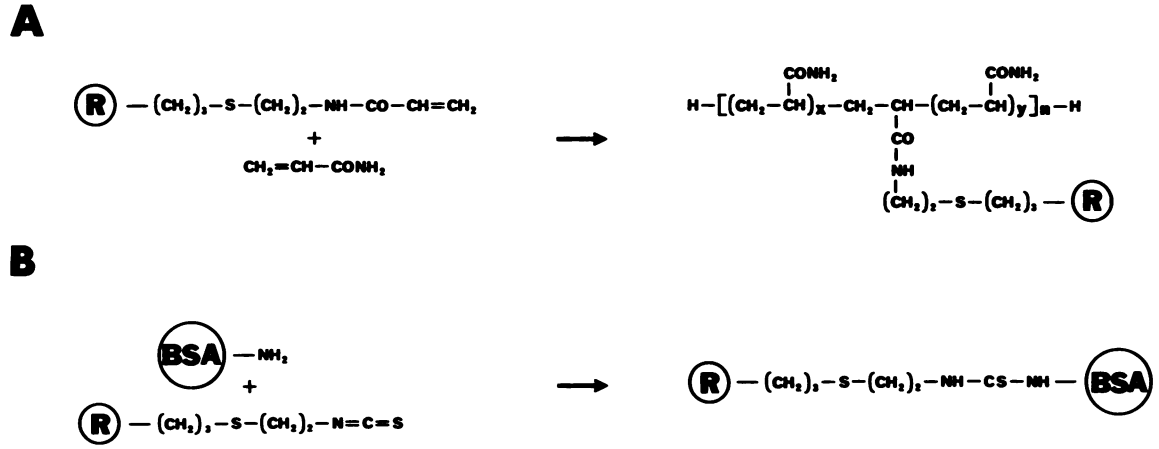

FIG. 1. Chemical structures of polyacrylamide derivatives (A) and BSA glycoconjugates (B) containing the carbohydrate ligand R. For the chemical structures of R, see Table 1.

raphy on plates of silica gel 60 (Merck) with chloroform-methanol-water (10/10/3 by volume) as the irrigant and detection with UV light (254-nm wavelength). When the reaction was completed, usually within 3 h of stirring at room temperature, the organic phase was removed and the aqueous layer was extracted three times with 3 ml of chloroform. The aqueous phase was freed from residual chloroform by evaporation under a stream of nitrogen and added to a solution of BSA (10 mg, essentially fatty acid free; Sigma) in 0.3 M sodium chloride containing 0.1 M sodium bicarbonate (1 ml). The reaction mixture was stirred for 48 h at room temperature and then separated by gel permeation chromatography with a 35- by 1.6-cm column of Sephadex G-50 and 10 mM sodium bicarbonate as the eluant. Collected ninhydrin-positive fractions were combined and dialyzed against phosphate-buffered saline (0.15 M, pH 7.2; PBS). Filter (0.22-μm pore size)-sterilized aliquots were stored at −20°C. The amounts of KDO present in the polyacrylamide derivatives and in the BSA conjugates were determined by using the thiobarbiturate assay after hydrolysis in acetate buffer (2), and the protein content was estimated by using commercial test kits.

(iv) Synthesis of amphiphilic partial structures of LPS. Sodium 3-deoxy-α-D-*manno*-2-octulopyranosylonate-(2→6)-2-deoxy-2-[(*R*)-3-hydroxytetradecanamido]-β-D-glucopyranosyl-(1→6)-2-deoxy-2-[(*R*)-3-hydroxytetradecanamido]-D-glucose and disodium [3-deoxy-α-D-*manno*-2-octulopyranosylonate-(2→4)-3-deoxy-α-D-*manno*-2-octulopyranosylonate]-(2→6)-2-deoxy-2-[(*R*)-3-hydroxytetradecanamido]-β-D-glucopyranosyl]-(1→6)-2-deoxy-2-[(*R*)-3-hydroxytetradecanamido]-D-glucose were synthesized as described previously (18, 25). These compounds are partial structures of LPS containing the glucosamine backbone of lipid A with two amide-linked 3-hydroxymyristic acid residues and one or two KDO residues and are abbreviated as KDO-GlcNhm$_2$ and KDO$_2$-GlcNhm$_2$, respectively.

Monoclonal antibodies. BALB/c mice were immunized by using the protocol of Ståhli et al. (30). Four mice were injected with an emulsion of 50 μg of KDO$_3$-GlcNAc-BSA in 125 μl of PBS mixed with an equal volume of Freund complete adjuvant (Difco). On day 0, four aliquots of 50 μl each were injected subcutaneously and 50 μl was applied intraperitoneally. On day 28, mice received a single intraperitoneal injection of 50 μg of antigen in 50 μl of PBS emulsified with an equal volume of Freund incomplete adjuvant (Difco). Eight days later, the mice were bled from the tail vein and the serum was tested for the presence of antibodies to the immunizing antigen in an enzyme immunoassay (EIA). The mouse with the highest titer received three booster injections of 200 μg each in 200 μl of PBS on days 56, 57, and 58. The first injection was given intravenously, and the others were given intraperitoneally. Two days after the last injection, the animal was exsanguinated and the spleen was removed. Spleen cells were prepared and fused at a ratio of 1:1 with mouse myeloma X63Ag8 cells by using polyethylene glycol 1500 (Boehringer Mannheim) according to conventional protocols. Hybridomas were screened by EIA with r595-207 LPS complexed with BSA as a solid-phase antigen. Relevant hybridomas were cloned twice by limiting dilution, adapted to serum-free medium by using Ultroser (GIBCO) or SF$_1$ medium (Costar) as a supplement, and isotyped (isotyper kit from Bio-Rad). Culture supernatants were prepared in 100-ml quantities, and the antibodies were purified on protein G-Sepharose (Pharmacia/LKB) as recommended by the supplier. The purification was ascertained by using polyacrylamide gel electrophoresis (PAGE) in the Laemmli system (19) with a 5% polyacrylamide stacking gel and a 12% polyacrylamide separating gel and then staining with Coomassie brilliant blue R, resulting in single bands for the light and heavy chains, respectively. Protein concentrations were determined by using the BCA assay (Sigma) and the Bradford assay (Bio-Rad) according to the instructions of the suppliers. Monoclonal antibodies A20 (immunoglobulin M) and A25 (immunoglobulin G3), which recognize a single α-pyranosidically linked terminal KDO residue and an α-2.4-linked KDO disaccharide, respectively (28), were used as controls, since they were expected to react similarly with all of the antigens used in this study. They were obtained in a purified form from B. J. Appelmelk, Amsterdam. The chlamydia-specific monoclonal antibodies S5-10 (5) and L2I-6 (9) were obtained after immunization with whole elementary bodies. They are specific for the KDO trisaccharide (5, 13).

Biotinylation of antibodies. Monoclonal antibody S25-23 (1 mg) was biotinylated by using a biotinylation kit (Amersham) according to the supplier's instructions.

Serology. (i) EIA. In the EIA, two types of antigen were used as the solid phase: LPS-BSA complexes and synthetic BSA-glycoconjugates were coated onto PolySorp and MaxiSorp microtiter plates (U-bottom plates; both from Nunc), respectively. The conditions described below were the same for both antigens; however, Tween 20 was omitted when LPS-BSA complexes were used. Unless stated otherwise, 50-μl volumes were used. Microtiter plates were coated with the respective antigen solution in 50 mM carbonate buffer (pH 9.2) at 4°C overnight. Plates were washed four times with PBS supplemented with 0.05% Tween 20 (Sigma) and 0.01% thimerosal (PBS-T); this buffer was used also in the following washing steps. Plates were blocked with PBS-T supplemented with 15% heat (56°C for 30 min)-inactivated bovine serum (PBS-TBS) for 1 h at 37°C on a rocker platform followed by two washing cycles. Appropriate antibody dilutions in PBS-TBS were added and incubated for 1 h at 37°C. After a wash, peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) or M (IgM) (heavy chain specific; Dianova) was added (diluted 1:500 to 1:1,000), and incubation was continued for 1 h at 37°C. After the plates were washed in PBS-T, they were washed twice in substrate buffer (0.1 M sodium citrate, pH 4.5). The substrate solution was freshly prepared as follows: 1 mg of azino-di-3-ethylbenzthiazolinsulfonic acid was dissolved in 1 ml of substrate buffer with sonication in an ultrasound water bath for 3 min, and then hydrogen peroxide (25 μl of a 0.1% solution) was added. After 30 min at 37°C, the reaction was stopped by the addition of 2% aqueous oxalic acid and the plates were read by using a microplate reader (Dynatech MR 700) at 405 nm. In competitive EIAs, twofold dilutions of unlabeled antibody (100 μl) were mixed with equal volumes of a dilution of biotinylated antibody; without the addition of competing antibody, this preparation had an A_{405} of 1.0. Aliquots (50 μl) of the mixture were added in duplicate to antigen-coated EIA plates. After incubation at 37°C for 1 h, peroxidase-labeled streptavidin (25 μl of a 1:10,000 dilution; Dianova) was added. Washing and the development of the reaction were as described above. Inhibition is expressed as the percentage of A_{405} obtained with PBS instead of the competing antibody.

(ii) Hemagglutination. Sheep erythrocytes (SRBCs) were washed three times in PBS. Packed cells (200 μl) were suspended in 5 ml of PBS and passively coated with various amounts of partially de-*O*-acylated LPS or synthetic partial structures (1 to 200 μg) at 37°C for 30 min with occasional

shaking. After the cells were washed in PBS, they were suspended at a final concentration of 0.2%. Serial twofold dilutions of antibody (50 μl) were made in V-shaped microtiter plates, and 50 μl of sensitized SRBCs was added. The plates were incubated at 37°C for 1 h and then left at 4°C for at least 4 h before 50% endpoint titers were read. In hemagglutination inhibition assays, 25-μl samples of appropriate antibody dilutions containing 3 to 4 hemagglutinating units were preincubated with various amounts of inhibitor in 25 μl of PBS at 37°C for 15 min, then 50 μl of antigen-coated SRBCs was added. Further incubation and reading were as described above.

(iii) Immunofluorescence. Confluent L929 monolayers, grown on glass coverslips (12 mm in diameter), in Iscove medium supplemented with 1% nonessential amino acids, 2 mM glutamine, 10% fetal calf serum, and gentamicin (10 μg/ml; all reagents from GIBCO) were infected with *C. trachomatis* serotype L2 or *C. psittaci* 6BC at a dose resulting in >80% infected cells within 24 to 36 h. The inoculation medium was composed as described above, but it contained only 2% fetal calf serum and added cycloheximide (1 μg/ml). Infected cells were fixed in cold (−20°C) methanol, washed in PBS, incubated with the respective monoclonal antibody, and stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antiserum (Dianova). The preparations were examined with a Zeiss fluorescence microscope with incident light.

(iv) Western blots. The BSA glycoconjugates (3 μg per lane) were separated by using PAGE on a 7.5% polyacrylamide gel and then stained with Coomassie brilliant blue R. For silver staining and Western immunoblots, amounts of conjugate equivalent to 0.15 nmol of carbohydrate ligand were applied to the gels. For Western blots with LPS as the antigen, r595-207 and R595 LPSs (1.6 μg per lane) were separated on a 15% polyacrylamide gel and then transferred to nitrocellulose. Monoclonal antibody A20 was used at a concentration of 1.5 μg/ml; all others were used at a concentration of 15 μg/ml. The reaction was developed with alkaline phosphatase-conjugated goat anti-mouse IgM and IgG, respectively, and 5-bromo-4-chloro-3-indoylphosphate *p*-toluidine and *p*-nitroblue tetrazolium chloride (Bio-Rad) as substrates.

(v) Dot blots. For dot blots, the mycobacteria in Freund complete adjuvant (Difco) were sedimented by centrifugation, washed successively in ether, acetone, and ethanol, lyophilyzed, and suspended in PBS at a concentration of 2 mg/ml. Aliquots (0.1 to 10 μg) were spotted on nitrocellulose, dried, and stained with monoclonal antibodies as described above for Western blots. Purified elementary bodies of *C. trachomatis* serotype L2 and *C. psittaci* 6BC were used in parallel.

RESULTS

Synthesis of glycoconjugates containing carbohydrate partial structures of chlamydial LPS. The tetrasaccharide KDO_3-GlcNAc, the trisaccharide KDO_3, the disaccharides α-KDO(2→8)-α-KDO and α-KDO(2→4)-α-KDO, and the α-KDO monosaccharide were synthesized and appropriately derivatized to allow covalent binding of high-molecular-weight carriers. The carbohydrate ligands were either copolymerized with acrylamide, yielding linear polyacrylamide derivatives, or coupled with BSA. In both cases, the ligand was incorporated by a spacer to allow free excess of antibodies tested (Fig. 1). The amount of ligand incorporated was determined by chemical analysis of the KDO content. In polyacrylamide derivatives, the range was 140 nmol/mg for the most bulky and thus the least reactive ligand, KDO_3-GlcNAc, to 660 nmol/mg for the α-KDO monosaccharide. In BSA conjugates, the range was 38 to 119 nmol/mg (Table 1). BSA conjugates were examined by using PAGE and staining (Fig. 2A and B) or by Western blot analysis (Fig. 2C and D). A monoclonal antibody recognizing a single KDO (27) reacted with all five antigens (Fig. 2C), whereas the KDO trisaccharide-specific antibody S25-23 (see below) only reacted with KDO_3-BSA and KDO_3-GlcNAc-BSA (Fig. 2D, lanes 4 and 5).

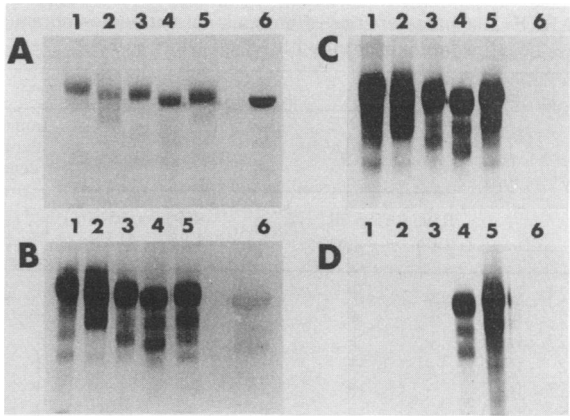

FIG. 2. PAGE of BSA glycoconjugates representing partial structures of chlamydial LPS. KDO-BSA (lane 1), 2.4-KDO_2-BSA (lane 2), 2.8-KDO_2-BSA (lane 3), KDO_3-BSA (lane 4), KDO_3-GlcNAc-BSA (lane 5), and BSA (lane 6) (3 μg of each) were separated on a 7.5% polyacrylamide gel and stained with Coomassie brilliant blue R (A). For silver staining (B) and Western blots with monoclonal antibody A20 (1.5 μg/ml) (C) or S25-23 (15 μg/ml) (D), samples of conjugate containing 0.15 nmol of carbohydrate ligand were applied to the gels.

Selection of monoclonal antibodies after immunization with synthetic KDO_3-GlcNAc-BSA. Mice were immunized with synthetic KDO_3-GlcNAc-BSA over 2 months as described in Materials and Methods. Hybridomas were screened for specific antibody production by EIA with R595 and recombinant r595-207 LPS complexed with BSA as well as synthetic KDO_3-GlcNAc-BSA as a solid-phase antigen. High concentrations of antigen were used to coat the EIA plates to facilitate detection of low-affinity antibodies. Two types of antibody specificity were obtained; antibodies of one type reacted with all antigens, and those of the other type reacted with r595-207 LPS and with KDO_3-GlcNAc-BSA but not with R595 LPS (Table 2). Antibodies of the latter type were chlamydia specific, whereas those of the former type recognized cross-reactive epitopes present in the LPSs of *S. minnesota* R595 and chlamydiae. As a further selection criterion, monoclonal antibodies were tested (i) in passive hemagglutination assays with SRBCs coated with R595 and r595-207 LPSs and (ii) in immunofluorescence assays with L929 monolayers infected with *C. psittaci* or *C. trachomatis*. Thus, we selected antibodies that recognized both isolated and membrane-associated LPSs. The monoclonal antibodies were cloned twice by limiting dilution, and isotypes were determined; all of the antibodies belonged to the IgG1 isotype except clone S25-7, which was an IgM. Thus, seven antibodies were obtained; together with three control antibodies, these seven are listed in Table 2. All antibodies reacted in dot blots with elementary bodies of *C. psittaci* and

TABLE 2. Characteristics of monoclonal antibodies obtained after immunization with KDO_3-GlcNAc-BSA

Antibody[a]	Isotype	Reactivity						
		EIA[b]			Hemagglutination[c]		Immunofluorescence[d]	
		R595 LPS	r595-207 LPS	KDO_3-GlcNAc BSA	R595 LPS	r595-207 LPS	L2	6BC
S23-24	IgG1	+	+	+	+	+	+	+
S25-7	IgM	+	+	+	+	+	+	+
S25-27	IgG1	−	+	+	−	+	+	+
S25-2	IgG1	−	+	+	−	+	+	+
S25-23	IgG1	−	+	+	−	+	+	+
S25-5	IgG1	−	+	+	−	+	+	+
S25-26	IgG1	−	+	+	−	+	+	+
A20	IgM	+	+	+	+	+	+	+
S5-10	IgG3	−	+	+	−	+	+	+
L2I-6	IgG3	−	+	+	−	+	+	+

[a] A20 is a monoclonal antibody recognizing an α-pyranosidically linked single KDO residue (28); S5-10 and L2I-6 are chlamydia-specific monoclonal antibodies obtained after immunization with elementary bodies of *C. psittaci* and *C. trachomatis*, respectively, recognizing the KDO trisaccharide (5).
[b] Complexes of LPS (R595 and r595-207) with BSA were used with the glycoconjugate KDO_3-GlcNAc-BSA as a solid-phase antigen.
[c] SRBCs coated with indicated antigen were used.
[d] L929 monolayers infected with *C. trachomatis* serotype L2 or *C. psittaci* 6BC were used.

C. trachomatis (1 μg per dot), but none reacted with the mycobacteria (up to 10 μg per dot) in the Freund complete adjuvant used for immunization (data not shown). Hybridomas were adapted to growth in serum-free medium, and monoclonal antibodies were purified by protein G-Sepharose affinity chromatography. Upon PAGE analysis, each antibody gave a single band for the light or heavy chain (data not shown). Immunoglobulins were stored as aliquots (1 mg/ml) at −20°C.

Determination of epitope specificities of monoclonal antibodies by hemagglutination and hemagglutination inhibition. The antibodies were tested in hemagglutination assays with R595 and r595-207 LPSs and KDO-$GlcNhm_2$ and KDO_2-$GlcNhm_2$ synthetic partial structures (data not shown). Monoclonal antibodies S23-24 and S25-7 had the same reactivity with R595 and r595-207 LPSs and the synthetic antigen KDO_2-$GlcNhm_2$ and no (S23-24) or very weak (S25-7) reactivity with KDO-$GlcNhm_2$. These data indicated that these two antibodies recognized the 2.4-linked KDO disaccharide. Since antibodies of this specificity were reported by Rozalski et al. (28) and since they were not useful for defining chlamydia specificity, they were not investigated further.

The remaining five monoclonal antibodies reacted exclusively with recombinant LPS r595-207 in concentrations between 0.06 and 0.63 μg/ml. The reaction pattern did not change with SRBCs coated with de-*O*-acylated or dephosphorylated LPS (data not shown). These monoclonal antibodies apparently recognized a carbohydrate epitope for which the 2.8-linkage between two KDO residues (not present in R595 LPS) was essential. To determine the minimal structure required for binding these clones, we studied hemagglutination inhibition assays with synthetic polyacrylamide antigens as inhibitors (Table 3). Monoclonal antibodies S25-5, S25-23, and S25-26 were inhibited to the same extent by KDO_3-polyacrylamide (PA) and KDO_3-GlcNAc-PA but not with KDO mono- or disaccharide antigens, even at 300-fold-higher concentrations. Therefore, these monoclonal antibodies bound the KDO trisaccharide as the minimal structure. S25-2 and S25-27 exhibited a more complicated reaction pattern. Both antibodies were inhibited similarly by 2.8-KDO_2-PA, KDO_3-PA, and KDO_3-GlcNAc-PA but also by 2.4-KDO_2-PA and, in the case of S25-27, by KDO-PA, although 5 to 10 times less effectively. A similar pattern was observed in Western blots with glycoconjugates as antigens (data not shown). However, when LPS was separated by PAGE and transferred to nitrocellulose, S25-2 and S25-27 reacted in a chlamydia-specific way; i.e., they bound to r595-207 LPS but not to R595 LPS (Fig. 3).

TABLE 3. Inhibition of monoclonal antibodies with synthetic polyacrylamide antigens in passive hemagglutination[a]

Inhibitor[b]	Inhibitor concn (pmol/ml)[c] yielding 50% inhibition with antibody:				
	S25-23	S25-5	S25-26	S25-2	S25-27
KDO-PA	>500	>500	>500	>500	1.80
2.4-KDO_2-PA	>500	>500	>500	2.34	2.34
2.8-KDO_2-PA	>500	>500	>500	0.78	0.59
KDO_3-PA	1.56	7.00	1.17	0.59	0.25
KDO_3-GlcNAc-PA	1.56	7.00	1.17	0.59	0.25

[a] SRBCs coated with recombinant r595-207 LPS were used.
[b] See Table 1 and Fig. 1 for chemical structures.
[c] Means of at least two values not differing by more than one dilution step.

Determination of epitope specificities by EIA. Antibodies were tested in a solid-phase EIA by checkerboard titrations with KDO-BSA, 2.4-KDO_2-BSA, 2.8-KDO_2-BSA, KDO_3-BSA, and KDO_3-GlcNAc-BSA as antigens. The range of antibody concentrations was from 5 to 5,000 ng/ml, and the antigen concentration ranged from 0.2 to 30 pmol of carbohydrate ligand per well. The lowest antibody dilution yielding an optical density of >1.0 with the respective antigen (1.9 pmol of carbohydrate ligand per well) is given in Table 4. At this low epitope density, the most distinct reactivity pattern was observed with high-affinity antibodies. Monoclonal an-

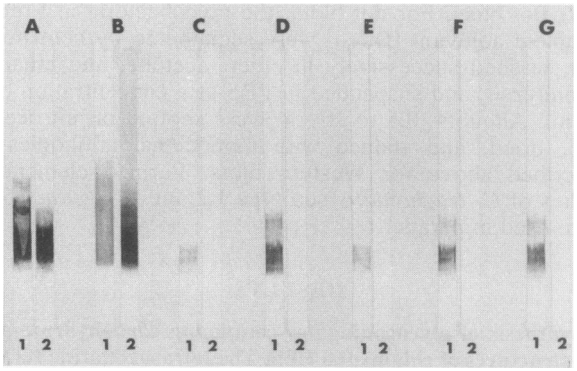

FIG. 3. Reactivity of monoclonal antibodies with LPS in Western blots. LPSs r595-207 (lane 1) and R595 (lane 2) (1.6 μg of each) were separated by PAGE (15% polyacrylamide), transferred to nitrocellulose, and reacted with monoclonal antibodies. The cross-reactive monoclonal antibodies were A20 (A) and A25 (B), which recognize a single α-pyranosidically linked KDO residue and the α-2.4-linked KDO disaccharide, respectively (27, 28). The chlamydia-specific antibodies were the KDO_3-specific monoclonal antibodies S25-5 (C), S25-23 (D), and S25-26 (E) and the 2.8-KDO_2-reactive monoclonal antibodies S25-2 (F) and S25-27 (G). All antibodies were used at 15 μg/ml except A20, which was used at 1.5 μg/ml.

TABLE 4. EIA checkerboard titrations of monoclonal antibodies against artificial antigens (BSA conjugates)[a]

Antigen[b]	Lowest concn (ng/ml) of indicated antibody yielding an A_{405} of ≥1.0[c]							
	S25-2	S25-27	S25-23	S25-5	S25-26	S5-10	L2I-6	A20
KDO-BSA	5,000	5,000	>5,000	>5,000	>5,000	>5,000	>5,000	4.8
2.4-KDO_2-BSA	2,500	5,000	>5,000	>5,000	>5,000	>5,000	>5,000	4.8
2.8-KDO_2-BSA	625	1,250	>5,000	>5,000	>5,000	>5,000	>5,000	4.8
KDO_3-BSA	625	2,500	9.8	78	78	2,500	2,500	2.4
KDO_3-GlcNAc-BSA	312	1,250	19.5	39	78	1,250	2,500	4.8

[a] Twofold dilutions of antibody, starting with 5 μg/ml, were tested against twofold dilutions of antigen, starting with 30 pmol of carbohydrate ligand per well.
[b] See Table and Fig. 1 for chemical structures.
[c] The data listed are those obtained with 1.9 pmol of carbohydrate ligand per well.

tibodies S25-5, S25-23, and S25-26 reacted with KDO_3-BSA and KDO_3-GlcNAc-BSA with differences not exceeding one dilution, and they were not reactive with the KDO disaccharide and monosaccharide antigens. Monoclonal antibodies S5-10 and L2I-6, obtained after immunization with elementary bodies of *C. psittaci* and *C. trachomatis*, respectively, exhibited a similar reaction pattern but at much higher concentrations. The specificity of this reaction was shown by using higher epitope densities (30 pmol of carbohydrate ligand per well). Under these conditions, clones S5-10 and L2I-6 bound equally to KDO_3-BSA and KDO_3-GlcNAc-BSA with 39 and 78 ng/ml, respectively, and to 2.8-KDO_2-BSA with 5 μg/ml.

Monoclonal antibodies S25-2 and S25-27 gave similar reactivities with 2.8-KDO_2-BSA, KDO_3-BSA, and KDO_3-GlcNAc-BSA as antigens. They also reacted with KDO-BSA and 2.4-KDO_2-BSA, although to a significantly lesser extent. To exclude the possibility that these reactivities were caused by different coating efficiencies of the glycoconjugates, the monoclonal antibody A20 (28), which recognized a single α-pyranosidically linked KDO residue, was used as a positive control. Similar reactivities of A20 with all five antigens indicated that the glycoconjugate epitopes were similarly exposed after immobilization on the plates. Also, in Western blots A20 was able to bind to all antigens tested (Fig. 2B). Therefore, the minimal structure required for binding was the KDO trisaccharide for clones S25-5, S25-23, and S25-26 and the 2.8-linked KDO disaccharide for monoclonal antibodies S25-2 and S25-27.

Comparison of KDO trisaccharide-specific monoclonal antibodies. Antibody binding curves were determined in the EIA by testing dilutions of monoclonal antibodies against 2 pmol of ligand per well (Fig. 4). The highest affinity was observed with clone S25-23, which still reacted at 2 ng/ml. Monoclonal antibodies S25-5 and S25-26 were eight times less reactive. However, S5-10 and L2I-6 were at least 2 orders of magnitude less reactive than clone S25-23. Similar results were obtained when KDO_3-GlcNAc-BSA instead of KDO_3-BSA was used as a solid-phase antigen (data not shown).

Monoclonal antibody S25-23 was used in a competitive binding assay. The binding of biotinylated S25-23 was inhibited with different trisaccharide-specific antibodies. With 30 pmol of KDO_3-BSA per well as a solid-phase antigen, inhibition with unlabeled S25-23 served as a positive control. This experiment also showed that the binding was not changed after biotinylation. Binding of biotinylated S25-23 was completely inhibited with its unlabeled counterpart and with all other monoclonal antibodies, thus indicating that they react with the same or at least an overlapping epitope (Fig. 5). However, the antibody concentrations required for 50% inhibition varied from 300 ng to 5 μg/ml, confirming the differences in affinity described above (Fig. 4).

DISCUSSION

Bacteria of the genus *Chlamydia* share a common LPS antigen, known as the group-specific antigen, that is active in complement fixation assays to determine antibodies against chlamydiae (10). Although the LPS is a major surface antigen, its molecular structure remained obscure for many years. The combination of molecular genetics (22, 31), hybridoma technology (9), and analytical (1, 3, 23) and synthetic (15, 17) chemistry enabled the structural and antigenic analysis of the chlamydial LPS antigen. The carbohydrate backbone of the LPS is composed of the pentasaccharide shown in Fig. 6. The glucosamine disaccharide moiety of LPS represents the dephosphorylated lipid A backbone, a common architectural principle of gram-negative LPS. The 2.4-linked disaccharide portion of the KDO trisaccharide, present in other LPSs (3), explains the reported (5, 7–9, 23, 24) cross-reactivity between chlamydial and enterobacterial LPSs of the Re chemotype. The 2.8 linkage between two KDO residues is not present in other bacterial LPS structures. Therefore, this structure is involved in the chlamydia-specific epitope. After mice are immunized with elementary bodies of *C. psittaci* (5) and *C. trachomatis* (9), selected monoclonal antibodies recognize the genus-specific epitope. Through chemical synthesis of glycoconjugate antigens composing the complete pentasaccharide and partial structures, the KDO trisaccharide was

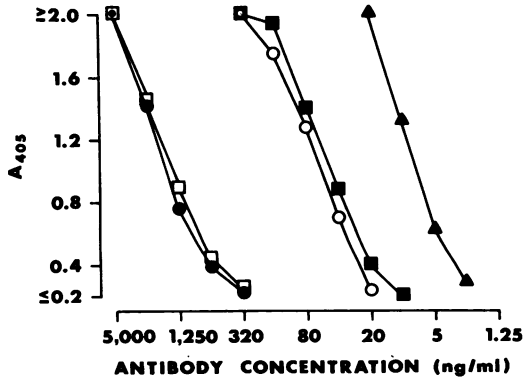

FIG. 4. Binding curves of chlamydia-specific monoclonal antibodies. Serial dilutions of S25-5 (■), S25-23 (▲), S25-26 (○), S5-10 (□), and L2I-6 (●) were reacted with synthetic KDO_3-BSA (2 pmol of carbohydrate ligand per well) in a solid-phase EIA.

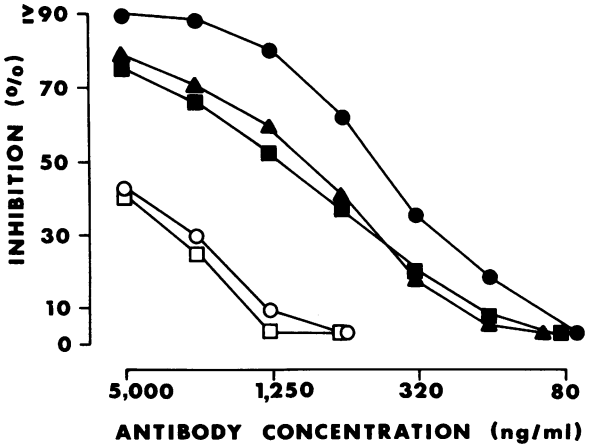

FIG. 5. Competitive solid-phase EIA of KDO trisaccharide-specific monoclonal antibodies. A constant amount of biotinylated S25-23 was incubated with serial dilutions of unlabeled S25-5 (▲), S25-23 (●), S25-26 (■), S5-10 (○), and L2I-6 (□) with KDO_3-BSA (30 pmol of carbohydrate ligand per well) in a solid-phase EIA. The percent inhibition of the biotinylated antibody is indicated on the ordinate.

found to be the minimal structure allowing binding of chlamydia-specific antibodies (13).

In the present study we asked two questions: (i) are synthetic glycoconjugates, which are assumed to represent the complete genus-specific epitope, able to induce antibody specificities similar to those observed after immunization with bacteria, and (ii) is the 2.8-linked KDO disaccharide the smallest structure of the trisaccharide to confer chlamydia specificity? The answer to both questions is yes. After mice were immunized with synthetic KDO_3-GlcNAc-BSA, we

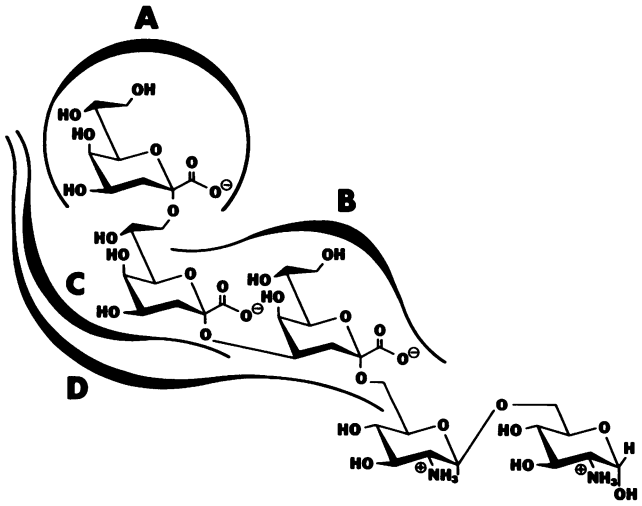

FIG. 6. Schematic representation of epitope specificities of monoclonal antibodies against the carbohydrate backbone of chlamydial LPS. Antibodies of types A and B recognize a single α-pyranosidically linked KDO residue and the α-2.4-linked KDO disaccharide, respectively. They are raised after contact with chlamydiae or Re-type bacteria and react with chlamydial and Re-type LPS. Antibodies of types C and D bind to the 2.8-linked KDO disaccharide and the KDO trisaccharide, respectively. They are induced only after contact with chlamydiae and do not react with Re-type or other LPS and are thus chlamydia specific.

selected monoclonal antibodies that were able to react (i) in an EIA with isolated recombinant LPS r595-207 and synthetic BSA-glycoconjugate antigens, (ii) in a hemagglutination assay with recombinant LPS, (iii) in a hemagglutination inhibition assay with synthetic polyacrylamide derivatives containing different carbohydrate ligands, (iv) in an immunofluorescence assay with chlamydia-infected L929 cells, and (v) in Western blots with glycoconjugates and LPS as antigens. Since it is known that the physicochemical environment critically determines the exposure of carbohydrate epitopes in amphiphilic LPS antigens (4), different serological tests were chosen to show that the antibodies investigated were able to react with LPS in distinct physicochemical surroundings such as the association with the membrane of elementary bodies or SRBCs or complexing with BSA and binding to polystyrol surfaces. Monoclonal antibodies fulfilling these prerequisites were delineated as chlamydia specific (in structural terms, requiring the 2.8-linked KDO disaccharide for binding) or as reacting also with Re mutant LPS. The latter monoclonal antibodies recognized the 2.4-linked KDO disaccharide. Because such antibodies were obtained and characterized after immunization with Re mutant bacteria (28), they were not investigated further in our study. The remaining five monoclonal antibodies were differentiated into those recognizing the 2.8-linked KDO disaccharide (S25-2 and S25-27) or those recognizing the KDO trisaccharide (S25-5, S25-23, and S25-26). The latter antibodies were specific for the KDO trisaccharide. The disaccharide-reactive antibodies had an absolute requirement for the 8-substituted KDO in the hemagglutination test (Table 2) and in Western blots with LPS as the antigen (Fig. 3). However, they reacted in inhibition assays to some extent with the 2.4-linked KDO disaccharide, and S25-27 also reacted with the KDO monosaccharide, when both antigens were presented as polyacrylamide derivatives (Table 3). In the EIA and Western blots (data not shown), the reactivity of S25-2 and S25-27 with glycoconjugates was not as strict as that of the trisaccharide-reactive antibodies. Although the antibodies bound preferentially to the 2.8-linked disaccharide, some reactivity was seen with the 2.4-linked disaccharide and KDO monosaccharide. These assay-dependent differences are probably related to the physicochemical presentation. When LPS is integrated into the SRBC membrane via its lipid A anchor, the flexibility of the surface-exposed KDO moiety is probably limited. In the inhibition assay, binding of the antibody to the competing inhibitor is facilitated by the spacer arm molecule in the polyacrylamide derivatives. In addition, the affinity of the disaccharide-reactive clones is significantly lower than that of the trisaccharide-reactive clones. Whereas the former bind in the EIA at concentrations ranging from 0.3 to 5 μg/ml, the best among the latter (S25-23) even binds at 2 ng/ml. Therefore, clones S25-2 and S25-27 are not able to define chlamydia specificity in all of the assays used. However, the data indicate that chlamydia specificity may also exist on the disaccharide level. Further experiments will show whether disaccharide-reactive and chlamydia-specific antibodies with higher affinity can be prepared.

We have compared the monoclonal antibodies against chlamydial LPS described in this study with those reported earlier. Immunization with the synthetic glycoconjugate antigen KDO_3-GlcNAc-BSA is able to induce antibodies with the same epitope specificity as those observed after immunization with whole elementary bodies. In addition, a broader spectrum of antibodies was induced with orders of magnitude higher in affinity. Figure 6 shows schematically

the binding domains for each of the antibody specificities in the carbohydrate backbone of chlamydial LPS. Also illustrated are the antibody binding sites against KDO structures present in other LPSs that cross-react with chlamydial LPS.

With this study, we have fulfilled for the characterization of bacterial surface antigens what Koch's postulates are for infectious diseases: identification of a bacterial surface structure as an antigen, determination of its chemical and antigenic structure, preparation and characterization of monoclonal antibody binding sites, chemical synthesis of the epitopes, and finally proof that the synthetic product has the same antigenic and immunogenic properties as its natural counterpart.

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