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DETERMINATION OF THE EPITOPE SPECIFICITY OF MONOCLONAL ANTIBODIES AGAINST THE INNER CORE REGION OF BACTERIAL LIPOPOLYSACCHARIDES BY USE OF 3-DEOXY-D-*manno*-OCTULOSONATE-CONTAINING SYNTHETIC ANTIGENS*

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ABSTRACT

Partial structures of enterobacterial lipopolysaccharides (LPS) of the Re-chemotype, consisting of lipid A and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), as well as oligosaccharides and derivatives of Kdo were synthesized and used to characterize the epitope specificity of monoclonal antibodies against Re-mutant LPS. High-molecular-weight antigens, obtained after copolymerization of the respective allyl glycosides with acrylamide, and the haptenic oligosaccharides were used in immunoprecipitation, immune hemolysis, and in inhibition assays. A monoclonal antibody (clone 20, IgM) recognizing a terminal Kdo*p* group was shown to require for its binding the α-anomeric configuration and OH-4 and OH-5 groups, whereas the C-7–C-8 chain was of minor importance. Another monoclonal antibody (clone 25, IgG_3), which recognizes a (2→4)-linked Kdo disaccharide, was

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shown to require for its binding the α-anomeric configuration of both residues. The isomer having a reducing β-Kdo residue was significantly less active, and that with a terminal β-Kdo group was completely inactive. The OH-5 group of the reducing residue was shown to be not important for the specificity of this antibody, since it could be replaced by a hydrogen atom without loss of serological reactivity. The α-(2→8)-linked Kdo disaccharide was strongly cross-reactive with its (2→4)-linked isomer. The antibody recognized also parts of the 2-amino-2-deoxy-D-glucose backbone of lipid A.

INTRODUCTION

Lipopolysaccharides (LPS) are surface components of the gram-negative cell wall. LPS are composed of a lipid part, termed lipid A, and a heteropolysaccharide which, in enterobacteria and many other bacterial families, is subdivided into the core oligosaccharide and the O-specific chain. Besides their physiological role for the integrity of the bacterial outer membrane, LPS induce numerous pathophysiological functions in higher organisms. Therefore, the LPS are also termed endotoxins[1]. In addition, LPS exhibit immunoreactivity, *i.e.*, they are immunogens and antigens, a property to which expression is given by the term O-antigen[2].

We are interested in the immunoreactivity of LPS with regard to the potential use of these antigens and the corresponding antibodies for active or passive immunoprophylaxis of gram-negative infection, currently a severe problem in clinical medicine. All three regions, lipid A, core, and O-chain exhibit distinct immunoreactive properties in higher mammalians. The O-chain determines serotype specificity in serological and chemical terms, and is, thus, of limited value for immunoprophylaxis. Both the core oligosaccharide and the lipid A component are antigenic structures which are similar in LPS from various gram-negative bacteria. Since lipid A antigenicity and immunogenicity are cryptic in LPS, being exposed only after removal of the polysaccharide components, special attention has been paid to antigens of the core region. Since the chemical structure of this region has been elucidated only recently, there was no molecular basis for the understanding of how antibodies interact with the unique carbohydrates of this region, *i.e.*, heptoses and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo). Based on the structural knowledge of the Kdo region established[3–8] by our group in recent years, we have prepared monoclonal antibodies against the Kdo region of LPS from Re mutants of Enterobacteria, the saccharide portion of which is composed only of the disaccharide α-Kdo*p*-(2→4)-α-Kdo*p*-(2→).

In a previous study, we reported[9] on two monoclonal antibodies recognizing, in LPS of *Salmonella minnesota* rough mutants, specifically a terminal α-linked pyranosidic Kdo group and the α-(2→4)-linked Kdo disaccharide, respectively. Now, we have synthesized numerous Kdo oligosaccharides to characterize further the epitope specificity of these monoclonal antibodies and we report herein the results obtained.

EXPERIMENTAL

Bacterial LPS. — The LPS were extracted by the phenol–chloroform–petroleum ether method[10] from *Salmonella minnesota* rough mutant chemotype Re (strain R595) and *Escherichia coli* chemotype Re (strain F515). They were purified by repeated ultracentrifugation, followed by conversion into the uniform triethylammonium salt after electrodialysis[11]. Alkali-treated LPS (LPS-OH) was prepared with 0.25M NaOH at 56° for 2 h, followed by precipitation with ethanol. Smith-degraded LPS ($LPS_{ox/red}$) was prepared from Re LPS of *S. minnesota* and *E. coli* in the following way: LPS (10 mg) was dissolved in water (1 mL) and 0.2M $NaIO_4$ (100 μL) was added, followed by magnetic stirring in the dark at 4° for 5 d. After the addition of 1,2-ethanediol (50 μL), $NaBH_4$ (20 mg) was added and the mixture was kept at room temperature for 1 h. After dialysis against water, the retentate was acidified with acetic acid to pH 3.5, kept for 1 h at 37°, and evaporated several times to dryness after the addition of methanol. Chemical analysis by the thiobarbiturate assay[12] for 3-deoxyaldulosonic acids indicated that 50% of the reactivity present in LPS had been destroyed, and 3-deoxyheptulosonic acid was identified as the main component (data not shown) by g.l.c.–m.s. of the reduced and methylated products obtained after hydrolysis as described[13].

Synthetic antigens. — Sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosid)onate[14] (**2**), sodium (allyl 3-deoxy-β-D-*manno*-octulopyranosid)onate[14] (**4**), sodium (allyl 3,5-dideoxy-α-D-*arabino*-octulopyranosid)onate[15] (**6**), sodium (allyl 3,5-dideoxy-β-D-*arabino*-octulopyranosid)onate[15] (**8**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→4)-sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosid)onate[14] (**10**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→4)-sodium (allyl 3-deoxy-β-D-*manno*-octulopyranosid)onate[16] (**13**), *O*-(sodium 3-deoxy-β-D-*manno*-octulopyranosylonate)-(2→4)-sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosid)onate[16] (**15**), *O*-(sodium 3-deoxy-β-D-*manno*-octulopyranosylonate)-(2→4)-sodium (allyl 3-deoxy-β-D-*manno*-octulopyranosid)onate[16] (**17**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→8)-sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosyl)onate[17] (**19**), *O*-(sodium 3-deoxy-β-D-*manno*-octulopyranosylonate)-(2→8)-sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosid)onate[17] (**21**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→4)-sodium (allyl 3,5-dideoxy-α-D-*arabino*-octulopyranosid)onate[15] (**23**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→7)-sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosid)onate[18] (**25**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→4)-*O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→4)-sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosyl)onate[19] (**27**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→4)-*O*-(sodium 3-deoxy-β-D-*manno*-octulopyranosylonate)-(2→4)-sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosid)onate[19] (**29**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→8)-*O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→4)-sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosid)onate[17] (**31**), *O*-(sodium 3-deoxy-α-D-*manno*-octulo-

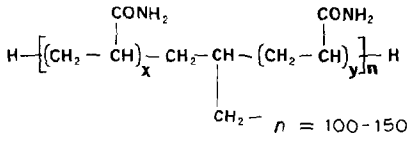
pyranosylonate)-(2→6)-*O*-(2-deoxy-2-amino-β-D-glucopyranosyl)-(1→6)-2-deoxy-2-amino-D-glucose[20] (**33**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→6)-*O*-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-(1→6)-2-deoxy-2-acetamido-D-glucose[20] (**34**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→6)-*O*-{2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino]-β-D-glucopyranosyl}-(1→6)-2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino]-D-glucose[21] (**35**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→4)-*O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→6)-*O*-(2-deoxy-2-amino-β-D-glucopyranosyl)-(1→6)-2-deoxy-2-amino-D-glucose[22] (**36**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→4)-*O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→6)-*O*-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-(1→6)-2-deoxy-2-acetamido-D-glucose[22] (**37**), *O*-(sodium 3-deoxy-α-D-*manno*-octulopyranosylonate)-(2→6)-*O*-{2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino]-3-[(*R*)-3-hydroxytetradecanoylamino]-β-D-glucopyranosyl hydrogen 4-phosphate}-(1→6)-2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino]-3-[(*R*)-3-hydroxytetradecanoylamino]-D-glucose[23] (**38**) were synthesized as described in the respective reference. Copolymerisation of the allyl glycosides with acrylamide[14,24] yielded the high-molecular-weight antigens listed in Table I. In the

TABLE I

SYNTHETIC COPOLYMERISATION PRODUCTS AND THEIR INHIBITION VALUES IN THE PASSIVE-HEMOLYSIS ASSAY WITH MONOCLONAL ANTIBODIES CLONE 20 AND 25

Compound	*Structure*[a]	*Ligand*[b] *(nmol/mg)*	*Inhibition value obtained with clone*[c] *(pmol/mL)*	
			20	*25*
3	[α-KDO]$_n$-PA	323	0.6	3230
5	[β-KDO]$_n$-PA	502	>5000	>5000
7	[α-5d-KDO]$_n$-PA[d]	300	>5000	>5000
9	[β-5d-KDO]$_n$-PA[d]	400	>5000	>5000
11	[α-KDO-(2→4)-α-KDO]$_n$-PA	338	4.1	13.5
14	[α-KDO-(2→4)-β-KDO]$_n$-PA	281	3.4	354.1
16	[β-KDO-(2→4)-α-KDO]$_n$-PA	268	>5000	>5000
18	[β-KDO-(2→4)-β-KDO]$_n$-PA	273	>5000	>5000
20	[α-KDO-(2→8)-α-KDO]$_n$-PA	295	7.1	23.6
22	[β-KDO-(2→8)-α-KDO]$_n$-PA	382	4.6	>5000
24	[α-KDO-(2→4)-α-5d-KDO]$_n$-PA[d]	150	2	23
26	[α-KDO-(2→7)-α-KDO]$_n$-PA	146	18.3	365
28	[α-KDO-(2→4)-α-KDO-(2→4)-α-KDO]$_n$-PA	145	2.9	46.4
30	[α-KDO-(2→4)-β-KDO-(2→4)-α-KDO]$_n$-PA	129	5.2	162.5
32	[α-KDO-(2→8)-α-KDO-(2→4)-α-KDO]$_n$-PA	106	1.5	>5000

[a]PA = poly(acrylamide). [b]Determined by the thiobarbiturate assay after hydrolysis in 0.1M acetate buffer, pH 4.4 at 100° for 1 h. [c]The reaction of the respective monoclonal antibody with *S. minnesota* R595 LPS-coated sheep erythrocytes was inhibited by preincubation with the inhibitors listed; 2–3 hemolytic units of antibody were used. [d]Minimal values, since the exact amount could not be determined owing to the significantly lower molar extinction coefficient of 5-deoxy-Kdo; 5d = 5-deoxy.



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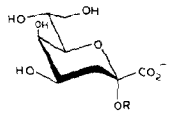

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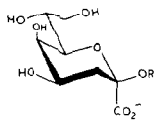

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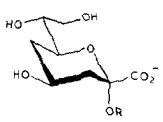

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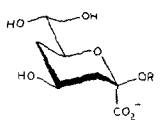

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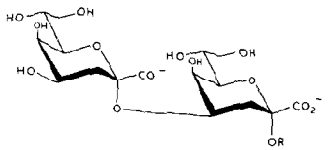
9 R = 1



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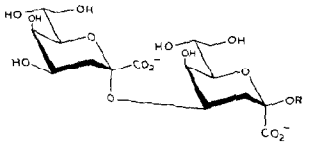

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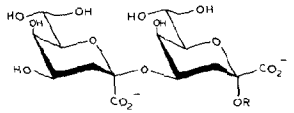

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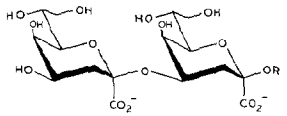

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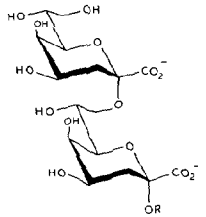

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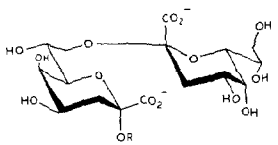

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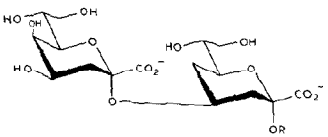

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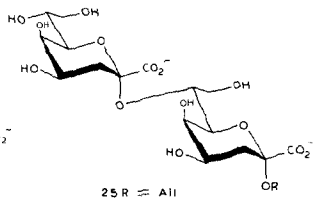

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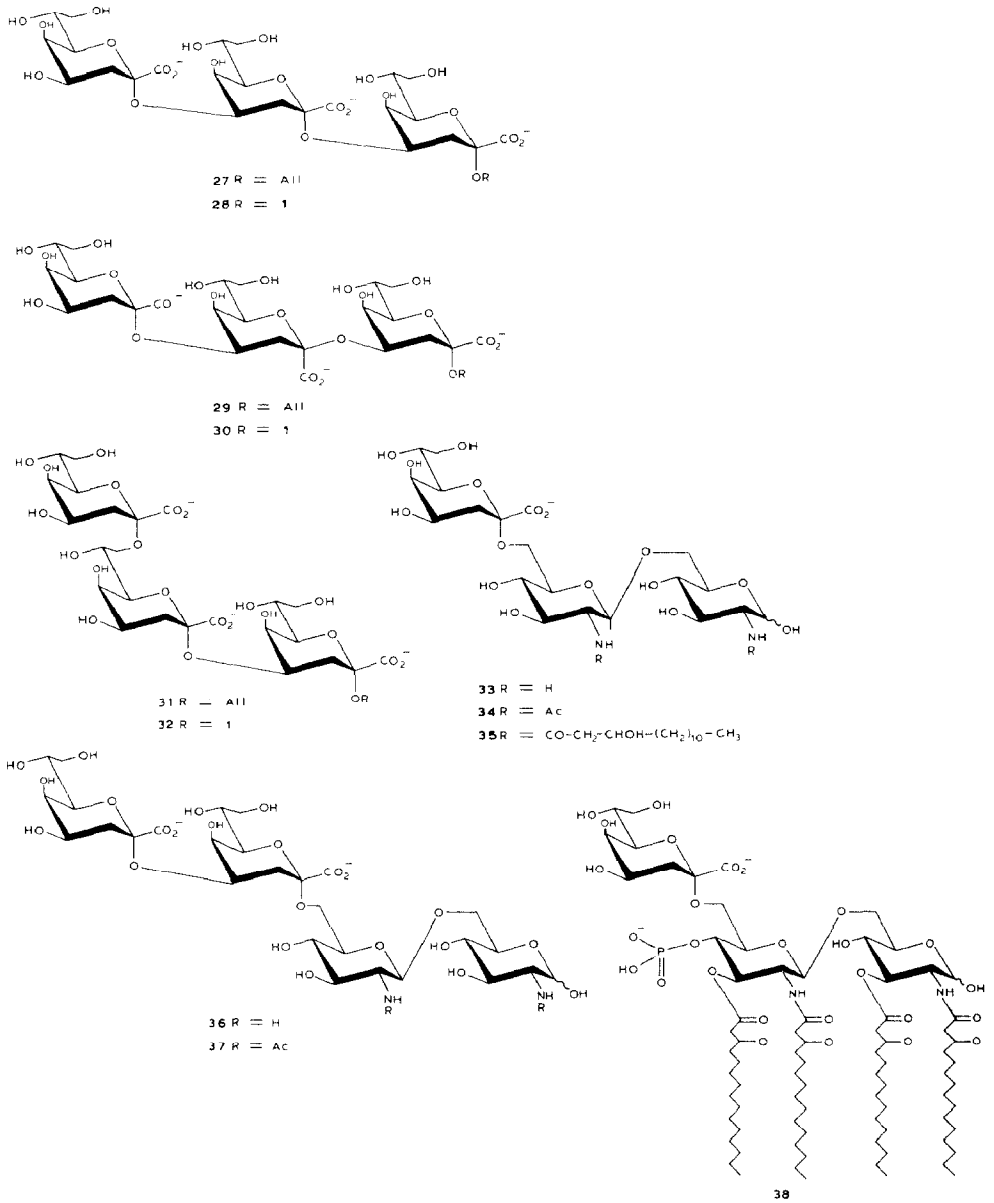

formula of **1**, n = 100–150, corresponding to mol. wts. of 60 000–100 000 (determined by gel-permeation chromatography using Sepharose 4B, Pharmacia/LKB). Compound **12** was prepared from **10** by condensation with cysteamine as described by Lee and Lee[25].

Immunoabsorbents. — Immunoabsorbents for affinity chromatography were prepared from **12** and **36** as follows. AH-Sepharose 4B (1 g) (Pharmacia/LKB) was

activated with 10% glutardialdehyde in 0.1M carbonate buffer (pH 9.2) for 1 h at room temperature. After the removal of excess glutardialdehyde by repeated cycles of washing and centrifugation, the ligand (2 mg) dissolved in the same buffer (2 mL) was added, and the reaction was allowed to proceed at room temperature overnight on an overhead shaker. To block free carbonyl groups, M glycine (0.1 mL) was added, and the mixture was kept for 1 h at room temperature. After extensive washing with distilled water, the Schiff base formed was reduced with $NaBH_4$ (~20 mg) for 1 h at room temperature. The absorbent was stored at 4° in phosphate-buffered saline solution (PBS) with NaN_3 (0.02%) and washed with PBS before use. Monoclonal antibody clones 20 and 25 were purified in the following way. The immunoabsorbent (3.5 mL of swollen gel in a small column) was loaded with crude ascites (1 mL, diluted 1:5 in PBS), and the effluent was tested for residual reactivity. The procedure was repeated until the absorbent was saturated. After washing with PBS (3 × 5 mL), elution was performed successively with 0.1M glycine–HCl (pH 3.2, 2.8, and 2.3; 5 mL each). Fractions (0.5 mL) were collected and the acid was neutralized with tris(hydroxymethyl)aminomethane. The fractions were tested for the presence of antibody by the passive-hemolysis assay and positive fractions (usually in the fractions eluted at pH 3.2 and 2.8) were combined. Both antibodies bound to either immunoabsorbent and were recovered by elution at low pH (2.8) in a yield of 80–90% based on the serological activity. The purified antibodies behaved similarly in serological assays to the crude ascites fluid; however, they tended to aggregate during storage.

Monoclonal antibodies. — The protocol for immunization and preparation of hybridomas has been described[26]. Briefly, Balb/c mice were immunized with heat-killed *S. minnesota* R595 bacteria by four intravenous injections during 42 d. Fusions were carried out three days after the last injection. Screening for the production of specific antibody was performed with *S. minnesota* R595 LPS by an enzyme-linked immunosorbent assay, and subcloning was carried out by conventional methods as was the production of ascites fluid and the determination of immunoglobulin isotypes and subgroups.

Serology. — The passive hemolysis test was used to determine antibody titers. Sheep erythrocytes (SRBC) (0.2 mL of washed, packed cells in 4 mL of PBS) were coated with graded amounts (1–200 μg) of LPS antigens at 37° for 30 min with shaking after 15 min. The sensitized cells were washed with PBS and suspended in veronal-buffered saline solution (VBS) at a concentration of 0.5%. Antibodies were serially diluted, and to 2 vols. of antibody were added 2 vols. of sensitized SRBC and 1 vol. of guinea-pig serum (diluted 1:20 in VBS) as a source of complement, followed by incubation at 37° for 1 h. The test was either carried out in 96 well microtiter plates (in which 1 vol. equals 25 μL) or in tubes (in which 1 vol. equals 250 μL). The former was read for 50% hemolysis by the naked eye, whereas the latter was read spectrophotometrically at 546 nm. One hemolytic unit of antibody is defined as the amount causing 50% of hemolysis. The antigenic relatedness of different antigens was determined by the passive-hemolysis-inhibi-

tion test, which was carried out in microtiter plates or in tubes. In the former, diluted antibody (25 μL) containing 2–3 hemolytic units was preincubated (15 min at 37°) with graded amounts of the respective inhibitor (0.1–400 pmol in a volume of 25 μL), followed by the addition of appropriately sensitized SRBC (50 μL) and complement (25 μL). The mixture was incubated at 37° for 1 h, and the plates were read for 50% inhibition of lysis by the naked eye. In the tube assay, 10-fold higher volumes were used and the antibody dilution was adjusted to give 80% of hemolysis by spectrophotometrical reading at 546 nm in a control without inhibitor. Inhibition was expressed as a percentage of this value.

Immunoprecipitation. — Quantitative immunoprecipitation was performed as follows: appropriately diluted antibody (~100 μg in 50 μL of PBS) was mixed in a small conical polypropylene tube (Eppendorf) with graded amounts of antigen (2–150 nmol in 50 μL of PBS) and incubated at 37° for 30 min. The formation of immunoprecipitates was allowed at 4° for 2 d, the sediments being resuspended twice per day. After centrifugation (10 000*g*, 30 min at 4°), the precipitates were washed twice with cold PBS, and then dissolved in 40mM NaOH (100 μL). Aliquots (40 and 20 μL) were used to determine the protein content with a commercial protein assay kit (Pierce), according to the supplier's instruction, with bovine serum albumin as a standard.

RESULTS

Characterization of monoclonal antibody clone 20 by immunoprecipitation. — Clone 20 (IgM) was prepared as ascites for quantitative immunoprecipitation assays using (α-KDO)$_n$-PA (**3**) and [α-KDO-(2→4)-α-KDO]$_n$-PA (**11**) as antigen, respectively. The resulting precipitation curves are shown in Fig. 1, indicating that both antigens yielded similar amounts of precipitated antibody in the equivalence

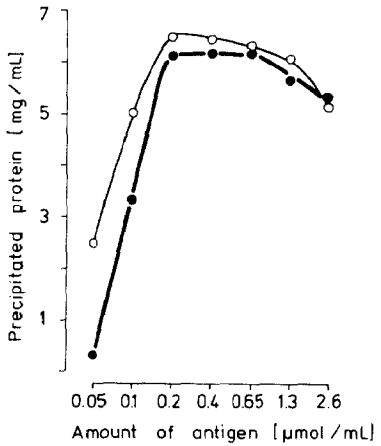

Fig. 1. Quantitative precipitation curve obtained with monoclonal antibody clone 20 (IgM) using (α-Kdo)$_n$-PA (—●—●—) and [α-Kdo-(2→4)-α-Kdo]$_n$-PA (—○—○—) as antigens.

zone. Analysis of the precipitates by poly(acrylamide)-gel electrophoresis under reducing conditions revealed that no other protein than immunoglobulin had been precipitated (data not shown). The rather flat peak maximum is characteristic for antibodies of the IgM isotype. When the molar ratios of antigen-binding sites (ten of which are present in an IgM molecule) to antigen ligand were calculated, the following results were obtained. With **11**, 50 nmol precipitated ~2.5 mg of IgM, which is equivalent to 2.8 nmol of IgM corresponding to 28 nmol of antigen-binding sites; thus a ratio of 1:1.8 was obtained. At the precipitation maximum, ~6.5 mg of immunoglobulin were precipitated with 200 nmol of ligand, corresponding to a molar antigen-binding site to ligand ratio of 1:2.8. With **3** as antigen, similar results were obtained in the equivalence zone, whereas in the range of antibody excess less antibody was precipitated. Since **3** contains more ligand equivalents per mg of copolymerization product than **11**, the data suggest that the degree of substitution is too high to allow interaction of each ligand with the bulky IgM molecule. For the same reason, the theoretical ratio of antigen-binding site to ligand of 1:1 may not be achieved.

Characterization of monoclonal antibody clone 25 by immunoprecipitation. — For the precipitation of clone 25 (IgG_3), only [α-Kdo-(2→4)-α-Kdo]$_n$-PA was used since this antibody did not react with Kdo monosaccharide. The precipitation curve obtained is displayed in Fig. 2. Since this antibody is of the IgG_3 isotype, the curve revealed a sharper precipitation maximum than the IgM antibody clone 20 (*cf.*, Fig. 1). At half-maximal precipitation, 1.45 mg of immunoglobulin were precipitated with 20 nmol of ligand, corresponding to a molar antigen-binding site to ligand ratio of 1:1.2. However, to reach the maximal antibody precipitation of

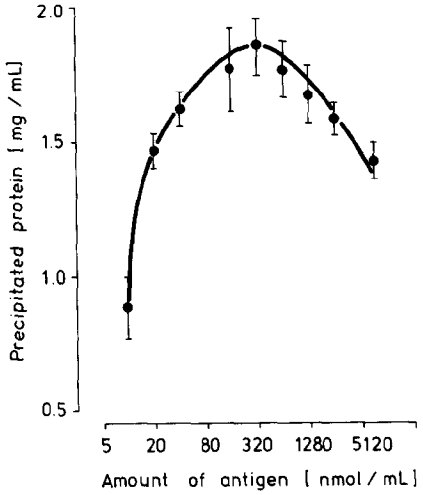

Fig. 2. Quantitative precipitation curve obtained with monoclonal antibody clone 25 (IgG_3) using [α-Kdo-(2→4)-α-Kdo]$_n$-PA as antigen.

1.87 mg/mL, 160 nmol of ligand were required, corresponding to a molar antigen-binding site to ligand ratio of 1:7.3.

Characterization of monoclonal antibody clone 20 by the passive hemolysis-inhibition assay. — The monoclonal antibodies were then investigated by inhibition studies using the complement-dependent, passive-hemolysis assay. The reactivity of the monoclonal antibody clone 20 with **35** was inhibited with synthetic copolymerization products listed in Table I. The system was adjusted to 2–3 hemolytic units, and the molar concentrations of the respective ligand yielding 50% inhibition were determined. As expected, compounds having a terminal α-linked Kdo group were efficient inhibitors with inhibition values in the range of 0.6–7 pmol/mL. The significantly higher value (18.3 pmol/mL) obtained with the (2→7)-linked Kdo disaccharide cannot be explained at present, but may be related to another accessibility of the nonreducing terminal group in this oligosaccharide. The two (2→8)-linked Kdo disaccharides (**20** and **22**) were equally effective inhibitors, although **22** contains a terminal β-Kdo group. Therefore, in this oligosaccharide, the reducing α-Kdo residue is the reactive part of the molecule, indicating that the C-7–C-8 side chain of the sugar is of minor importance for clone 20 specificity. Since the (2→4)-linked Kdo disaccharide antigen **16** has an α-Kdo residue at the reducing end but was inactive as an inhibitor, it is concluded that binding either requires a free OH-4 or is inhibited by steric hindrance of the substituent. In addition, OH-5 also seems to be important since the 5-deoxy-Kdo derivative **7** was inactive as inhibitor. The role of the C-7–C-8 chain of Kdo was further investigated by use of Smith-degraded LPS of the Re chemotype which contains an α-(2→4)-linked Kdo disaccharide as the only core constituent. Chemical analysis of the degraded product ($LPS_{ox/red}$) indicated that the terminal Kdo group had been

TABLE II

HEMOLYTIC-ANTIBODY TITERS OF MONOCLONAL ANTIBODIES CLONE 20 AND 25 WITH SRBC COATED WITH GRADED AMOUNTS OF LPS, SMITH-DEGRADED LPS, **35**, AND **38**

Antibody clone	*Antigen*	*Hemolytic-antibody titer obtained with SRBC coated with indicated antigen (μg/0.2 mL of packed cells)*					
		2	*8*	*32*	*64*	*128*	*200*
20	Ec F515 LPS	200	102,400	204,800	204,800	102,400	102,400
20	Ec F515 $LPS_{ox/red}$	<100	<100	51,200	102,400	102,400	102,400
20	Sm R595 LPS	51,200	102,400	102,400	204,800	204,800	102,400
20	Sm R595 $LPS_{ox/red}$	<400	<400	102,400	102,400	102,400	102,400
20	**35**	<400	51,200	102,400	204,800	204,800	102,400
20	**38**	400	102,400	204,800	204,800	102,400	102,400
25	Ec F515 LPS	<100	3,200	3,200	3,200	3,200	3,200
25	Ec F515 $LPS_{ox/red}$	<100	100	1,600	3,200	3,200	3,200
25	Sm R595 LPS	<100	800	1,600	3.200	3,200	3,200
25	Sm R595 $LPS_{ox/red}$	<100	<100	200	800	1,600	3,200
25	**35**	<100	<100	<100	<100	<100	<100
25	**38**	<100	<100	200	800	1,600	3,200

destroyed and that the lipid A-proximal Kdo unit had been oxidized to 3-deoxy-2-heptulosonic acid (shown by g.l.c.–m.s. after hydrolysis, reduction, and methylation; data not shown). $LPS_{ox/red}$ was used to sensitize erythrocytes which were then tested for their reactivity with clone 20. The results are depicted in Table II which shows that $LPS_{ox/red}$ is equally active as the native LPS with regard to the monosaccharide-reactive monoclonal antibody clone 20. Also, the absolute amounts of antigen used to sensitize the erythrocytes were comparable.

Characterization of monoclonal antibody clone 25 by the passive-hemolysis-inhibition assay. — In these experiments, the reactivity of clone 25 was tested with SRBC coated with LPS from *S. minnesota* R595. The synthetic counterpart **11** of the naturally occurring Kdo disaccharide gave an inhibition value of 13.5 pmol/mL (Table I). The isomer **14** having a β-Kdo residue at the reducing end was still active (inhibition value of 354 pmol/mL), though significantly less than **11**, whereas **16** and **18** carrying a terminal β-Kdo group were completely inactive at the concentrations tested (>5 nmol/mL). Surprisingly, the (2→8)-linked disaccharide antigen **20** was a good inhibitor (23.6 pmol/mL). The two trisaccharide antigens **28** and **30** yielded inhibition values of 46.4 and 162.5 pmol/mL, respectively, which was according to expectation. Obviously, the terminal α-(2→4)-α- and α-(2→4)-β-linked part of **28** and **30**, respectively, exhibit a similar conformation to the corresponding disaccharides in **11** and **14**. However, in the trisaccharide antigen **32**, the recognition of the terminal α-(2→8)-α-linked disaccharide portion is seemingly different from the disaccharide in **20**, since **32** was not active as an inhibitor (inhibition value >5 nmol/mL). The disaccharide glycoside **24**, carrying a 5-deoxy-Kdo residue at the reducing end, was also active, indicating that a free OH-5 in the reducing-end residue is not important for the interaction with clone 25.

To determine in how far the epitope expands into the 2-amino-2-deoxy-D-

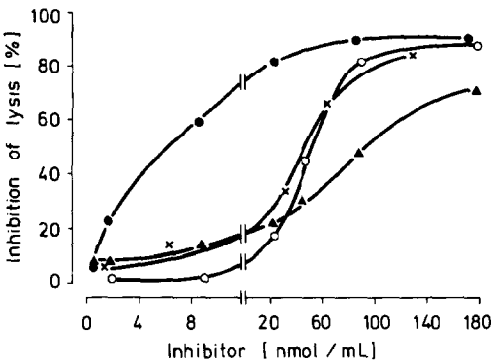

Fig. 3. Quantitative inhibition curves obtained in the hemolytic system of monoclonal antibody clone 25 and *S. minnesota* R595 LPS-coated sheep erythrocytes using the allyl glycosides of α-(2→4)-α- (—○—○—) and α-(2→8)-α-linked (—●—●—) Kdo disaccharides, the trisaccharide *O*-α-Kdo*p*-(2→6)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-2-acetamido-2-deoxy-D-glucose (—▲—▲—) and the tetrasaccharide *O*-α-Kdo*p*-(2→4)-*O*-α-Kdo*p*-(2→6)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→6)-2-acetamido-2-deoxy-D-glucose (—×—×—).

glucose region of the lipid A backbone in LPS, the trisaccharides **33** and **34**, and the tetraasaccharides **36** and **37** were synthesized. It was found that **33** and **36** were not active as inhibitors, presumably because of the formation of inner salts between the free amino groups of glucosamine and the carboxyl groups of Kdo (data not shown). When the amino groups were acetylated, whereby **34** and **37** were obtained, inhibition of clone 25 was observed. The results obtained with these two compounds in comparison with the allyl glycosides **10** and **19** in a hapten-inhibition assay are shown in Fig. 3. Interestingly, the (2→8)-linked allyl glycoside **19** was 5-fold more active than its isomer **10**, and the inhibition curve did not display the typical sigmoid shape as seen with the oher compounds. With the Kdo disaccharide glycoside **10** and the tetrasaccharide **37**, similar sigmoid inhibition curves were obtained yielding a 50% inhibition value of ~50 nmol/mL, indicating that, in the presence of the Kdo disaccharide, the 2-amino-2-deoxy-D-glucose disaccharide does not increase the interaction with antibody. Where only one Kdo group was present as in **34**, this structure still exhibited a significant, though lower, inhibition value. However, the inhibition curve was flattened and a 100% inhibition was not achieved even with 200 nmol/mL. The low binding capacity of clone 25 to **34** was further shown by a direct instead of an inhibition assay. For this purpose, the partial structure **38** of *E. coli* Re LPS was used in graded amounts to sensitize SRBC, which were then tested in the passive-hemolysis assay. The results are included in Table II. Since **38** contains a terminal α-linked Kdo group, the reactivity with clone 20 was used as a control for the sensitizing efficiency. The data show that clone 25 is able to lyse SRBC coated with **38**, only however when high amounts of antigen were used, leading to a high epitope density on the erythrocyte membrane. The monosaccharide-reactive antibody clone 20 lysed these cells already when low amounts of antigen were used. It should be noted, however, that IgM antibodies are more effective in complement-mediated lysis than IgG antibodies. Nevertheless, the data further confirm that although the main binding site for clone 25 is lying in the α-(2→4)-linked Kdo disaccharide of Re LPS, the Kdo-2-amino-2-deoxy-D-glucose region is also able to bind this antibody. The phosphate group at C-4 of the nonreducing 2-amino-2-deoxy-D-glucosyl group seemingly contributes to binding since the phosphate-less compound **35** did not react at all with clone 25.

DISCUSSION

The epitope specificities of the two monoclonal antibodies investigated can be summarized as follows. Clone 20 is specific for an α-linked Kdo residue. For the recognition by this antibody, the molecular requirements of the antigen include the anomeric region and the free OH-4 and OH-5. The primary OH-8 group does not participate at all in binding and that at C-7 is of minor, if any, relevance for the interaction with clone 20. Therefore, clone 20 seemingly binds to the α-pyranosidic ring of the Kdo residue. The role of the deoxy group, which is assumed to contribute significantly to hydrophobic interaction with antibody, thus could not be

tested. We have reported[27] on the occurrence of an octulosonic acid of unknown stereochemistry in the LPS of *Acinetobacter calcoaceticus*. Provided that the orientations at C-4 and C-5 are identical to those of the D-*manno*-Kdo, it will be interesting to compare the two possible isomers at C-3 of the octulosonic acid with regard to their interaction with clone 20.

Clone 25 binds mainly to the α-(2→4)-linked Kdo disaccharide portion of the Re LPS. It has been shown previously that it also binds to this disaccharide when it is present as a branch to the main polysaccharide chain in mutants other than Re. Since, in most LPS investigated, the disaccharide is not present, as a branch, in stoichiometric amounts, clone 25 binds only to those LPS carrying it in stoichiometric amounts, such as the LPS of *S. minnesota* Rb_2 chemotype[12]. Within the Kdo disaccharide, the terminal reducing Kdo residue contributes more to binding than does the nonreducing group since a change of the anomeric configuration of the latter decreased the reactivity, whereas that of the former abolished it completely. The OH-5 group of the reducing end residue is not involved in binding, since the replacement of it by a proton (5-deoxy-Kdo) did not reduce the immunoreactivity. Surprisingly, the α-(2→8)-linked Kdo disaccharide was only slightly less active, in inhibition assays, than its (2→4)-linked isomer when tested as copolymerization product, and even more active when tested as allyl glycoside. Although this reactivity was not expected, conformational analysis of the two disaccharide glycosides **10** and **19** revealed strong similarities between these two structures[28]. When, in addition to the Kdo disaccharide, the β-(1→6)-linked glucosamine disaccharide of the lipid A component of LPS is present, the binding of clone 25 is not significantly enhanced. If, however, a single α-linked Kdo is attached to the phosporylated 2-amino-2-deoxy-D-glucose disaccharide, the structure is still able to bind the antibody.

The present investigation contributes to understand the reactivity of how antibodies interact with carbohydrate constituents of the inner core region of bacterial LPS, and may also help to understand the molecular requirements for the binding of other proteins to this region, such as complement components, serum factors, bacteriophage receptors, or enzymes of the biosynthesis of this region.

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