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# Use of Synthetic Antigens to Determine the Epitope Specificities of Monoclonal Antibodies against the 3-Deoxy-D-*manno*-Octulosonate Region of Bacterial Lipopolysaccharide

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**Mouse monoclonal antibodies were raised against heat-killed bacteria of the Re mutant R595 of *Salmonella minnesota* and characterized by the passive hemolysis and passive hemolysis inhibition tests and by double immunodiffusion experiments using lipopolysaccharide (LPS) from different rough mutants of *S. minnesota* and synthetic antigens. The latter were copolymerization products of acrylamide with the α- and β-allylglycosides of 3-deoxy-D-*manno*-octulosonic acid (KDO) and the α-2,4-linked KDO disaccharide [poly-α-KDO, poly-β-KDO, and poly-(α-KDO)$_2$, respectively], and sodium (3-deoxy-D-*manno*-octulopyranosyl) onate-(2→6)-{2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino]-β-D-glucopyranosyl}-(1→6)-{2-deoxy-2-[(*R*)-3-hydroxytetrade-canoylamino]-D-glucose} [α-KDO-(GlcNhm)$_2$], representing a part structure of Re LPS. One antibody (clone 20, immunoglobulin M) was found to recognize a terminal α-linked KDO residue, since (i) it reacted in the passive hemolysis assay with α-KDO-(GlcNhm)$_2$ and all LPS tested, (ii) it was inhibited by all synthetic antigens containing α-linked KDO residues, and (iii) it gave a reaction of identity with poly-α-KDO and poly-(α-KDO)$_2$ in double immunodiffusion experiments. A second antibody (clone 25, immunoglobulin G3) was identified as specific for an α-2,4-linked KDO disaccharide, since (i) it reacted in immunodiffusion exclusively with synthetic poly-(α-KDO)$_2$ and not with the monosaccharide derivatives in either anomeric configuration, and (ii) it was inhibited only with poly-(α-KDO)$_2$ and with LPS from *S. minnesota* R595 (Re) and R345 (Rb$_2$). The reaction of this antibody with R345 LPS is attributed to the quantitative substitution with KDO disaccharide present as a side chain, which is not present in stoichiometric amounts in the other LPS.**

Lipopolysaccharides (LPS) are surface components of the gram-negative cell wall (26). In addition to their numerous physiological and pathophysiological functions, LPS are the major antigens of gram-negative bacteria (24, 25). The antigenicity and immunogenicity of LPS have attracted the interest of many investigators with regard to the potential use of these antigens and the corresponding antibodies for active or passive immunoprophylaxis of gram-negative infections.

LPS are amphipathic molecules composed of a lipid part, termed lipid A (31), which is responsible for the endotoxic activities of LPS (15), and a heteropolysaccharide, which is subdivided into the O-specific chain and the core oligosaccharide (26). All three regions, i.e., lipid A (7), core, and O chain (24, 25), are immunogenic in higher animals. The O chain exhibits serotype specificity in serological and chemical terms and is thus of limited value for immunoprophylaxis. Both the core oligosaccharide and the lipid A moiety are antigenic structures which are similar in LPS of 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.

Several groups have reported on the protective effects of antibodies against common core structures of LPS (1, 8–10, 20, 21, 35, 36). However, contradictory results were obtained by others (13, 17, 28, 32). These antibodies have been raised by immunization with the rough mutant strain R595 of *Salmonella minnesota* and the J-5 mutant of *Escherichia coli* (12). In addition, polyclonal and monoclonal antibodies have been assumed to recognize epitopes of the inner core region shared by many gram-negative bacteria belonging to various bacterial families (11, 18, 23, 27–30, 32). The epitope specificities of such monoclonal and polyclonal antibodies have not been determined on a molecular level in any previous study due to the lack of homogeneous and chemically defined antigens.

In recent years, we have investigated the chemical structure of the inner core region in enterobacterial LPS in order to understand the reactivities of monoclonal and polyclonal antibodies against it (3–6, 34). The structure of the 3-deoxy-D-*manno*-octulosonic acid (KDO) region is shown in Fig. 1. Synthetic antigens were prepared which were representative of the inner core region. By use of the defined antigens, we were able to characterize the epitope specificities of murine monoclonal antibodies against the inner core region, and here we report on the results obtained.

## MATERIALS AND METHODS

**Bacterial LPS.** LPS was extracted by the phenol-chloroform-petroleum ether method (16) from *S. minnesota* rough mutant chemotypes Rb$_2$ (strain R345), Rc (strain R5), Rd$_1$ (strain R7), Rd$_2$ (strain R4), and Re (strain R595). LPS was purified by repeated ultracentrifugation followed by conversion to the uniform triethylammonium salt after electrodialysis (14).

**Chemical analysis.** KDO was determined by the thiobarbiturate assay after release of KDO from LPS by different

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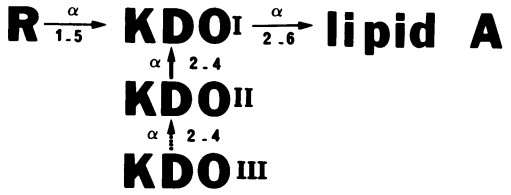


FIG. 1. Schematic structure of the inner core region of enterobacterial LPS. Re mutants contained only two KDO residues (KDO I and II), whereas mutants other than Re contained an additional KDO molecule (KDO III) in nonstoichiometric amounts (indicated by broken arrow) and the remaining core oligosaccharide (R).

hydrolysis conditions previously described (2). The protein content was determined by amino acid analysis. Aminosugars were identified by an amino acid analyzer and quantified by the Morgan-Elson reaction as modified by Strominger et al. (33).

**Synthetic antigens.** Sodium (allyl 3-deoxy-α-D-*manno*-octulopyranosyl)onate (poly-α-KDO), sodium (allyl 3-deoxy-β-D-*manno*-octulopyranosyl)onate (poly-β-KDO), and disodium [allyl 4-*O*-(3-deoxy-α-D-*manno*-octulopyranosyl)onate-3-deoxy-α-D-*manno*-octulopyranosyl]onate [poly-(α-KDO)$_2$] were synthesized (P. Kosma, J. Gass, G. Schulz, R. Christian, and F. M. Unger, Carbohydr. Res., in press) and subsequently copolymerized with acrylamide (10). The schematic structures of these products are shown in Table 1. In the formula in Table 1, $x + y = 18 \pm 2$, and $n$ = approximately 100 to 150, corresponding to molecular weights between 60,000 and 100,000. The synthesis of sodium (3-deoxy-α-D-*manno*-octulopyranosyl)onate-(2→6)-{2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino]-β-D-glucopyranosyl}-(1→6)-{2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino]-D-glucose} [α-KDO-(GlcNhm)$_2$] will be reported elsewhere (H. Paulsen and M. Schüller, manuscript in preparation). The chemical structure of this compound is shown in Fig. 2. Synthetic lipid A of *E. coli* (compound 506 [19]) and its 4′- and 1-dephospho derivatives (compounds 505 and 504, respectively [22]) were synthesized as described in the respective references; they were kindly provided by S. Kusumoto, Osaka, Japan.

**Monoclonal antibodies.** The protocol for immunization and fusion has been previously described in detail (B. J. Appelmelk, A. M. J. J. Verweij-van Vught, J. J. Maaskant, W. F. Shouten, L. G. Thijs, and D. M. MacLaren, FEMS Microbiol. Lett., in press). Briefly, BALB/c mice were immunized with heat-killed *S. minnesota* R595 bacteria by four intravenous injections during 42 days. Fusions were performed 3 days after the last injection. Screening was performed with *S. minnesota* R595 LPS, and subcloning was done by conventional methods, as was the production of ascites fluid and the determination of immunoglobulin isotypes and subgroups.

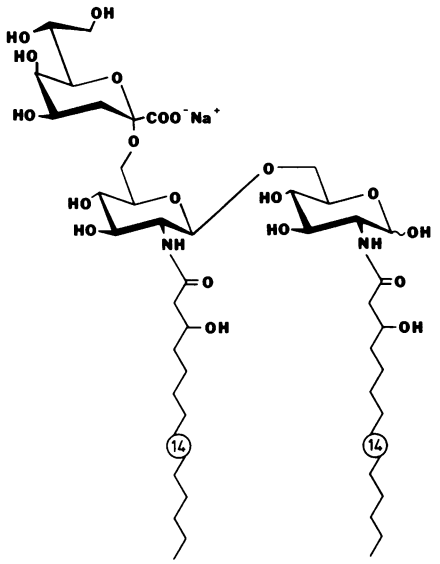


FIG. 2. Chemical structure of synthetic sodium (3-deoxy-α-D-*manno*-octulopyranosyl)onate-(2 → 6)-{2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino] - β-D-glucopyranosyl}- (1→6) -{2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino]-D-glucose}.

**Serology.** The passive hemolysis and passive hemolysis inhibition tests were performed as described previously (7), in which one hemolytic unit is the amount of antibody causing either 50% hemolysis or 50% inhibition of lysis, respectively. Double immunodiffusion was performed in 1% agarose in barbital-glycin-Tris buffer (pH 8.8; ionic strength, 80 mM) supplemented with sodium chloride (150 mM). Diffusion was allowed to proceed at room temperature for 24 h.

## RESULTS

Mouse monoclonal antibodies obtained after immunization with *S. minnesota* R595 were characterized for their epitope specificities by the passive hemolysis assay, double immunodiffusion experiments, and the passive hemolysis inhibition assay by using purified LPS from different rough mutants of *S. minnesota* and synthetic KDO-containing antigens.

**Chemical analysis of LPS.** The purified LPS had a protein content between 0.2 and 0.6% by weight as determined by amino acid analysis. The amount of KDO was determined by the thiobarbiturate assay by using different hydrolysis conditions (2) and is expressed on a molar basis relative to 2 mol of D-glucosamine. It was found that all LPS under investigation contained between two and three KDO residues per molecule (see Table 3). A considerable variation was found

TABLE 1. Synthetic copolymerization products of acrylamide with allylglycosides of KDO

| Compound[a] | Basic structure | R values |
|---|---|---|
| Poly-α-KDO | $CONH_2$ $CONH_2$ | Sodium (3-deoxy-α-D-*manno*-octulopyranosyl)onate |
| Poly-β-KDO | $H\text{-}[(CH_2\text{-}CH)_x\text{-}CH_2\text{-}CH\text{-}(CH_2\text{-}CH)_y]_n\text{-}H$ | Sodium (3-deoxy-β-D-*manno*-octulopyranosyl)onate |
| Poly-(α-KDO)$_2$ | $CH_2O\text{-}\mathbf{R}$ | Disodium 4-*O*-[(3-deoxy-α-D-*manno*-octulopyranosyl)onate]-3-deoxy-α-D-*manno*-octulopyranosyl)onate |

[a] Obtained by copolymerization of acrylamide (10) with the R-allyl derivatives. $x + y = 18 \pm 2$; $n$ = 100 to 150, corresponding to molecular weights between 60,000 and 100,000.

TABLE 2. Hemolytic titers of monoclonal antibodies with LPS from *S. minnesota* rough mutants

| Clone no. | Isotype | Hemolytic antibody titer obtained with[a]: | | | | | |
|---|---|---|---|---|---|---|---|
| | | LPS of *S. minnesota*: | | | | | Synthetic lipid A |
| | | R595 (Re) | R4 ($Rd_2$) | R7 ($Rd_1$) | R5 (Rc) | R345 ($Rb_2$) | |
| 14 | IgM | 51,200 | 40,960 | 51,200 | 51,200 | 51,200 | <20 |
| 20 | IgM | 204,800 | 25,600 | 102,400 | 20,480 | 819,200 | <20 |
| 25 | IgG3 | 12,800 | <20 | <20 | <20 | 5,120 | <20 |
| 27 | IgG3 | 640 | <20 | <20 | <20 | 1,280 | <20 |

[a] Antibodies were derived from ascites. Synthetic *E. coli* lipid A (compound 506) and its 4'- and 1-dephospho derivatives (compound 505 and 504, respectively) were used as previously described (7).

from one batch of LPS to another in strains R4, R7, and R5, in which values between 2.2 and 2.7 were recorded; the LPS of strain R595, however, contained constantly 2.1 KDO residues, and that of R345 contained 3 KDO residues. The neutral sugar composition did not vary (data not shown), and the amounts were similar to those reported earlier (3, 4).

**Passive hemolysis assay.** The hemolytic antibody titers against strain R595, R4, R7, R5, and R345 LPS (chemotypes Re, $Rd_2$, $Rd_1$, Rc, and $Rb_2$, respectively) of ascites prepared with the indicated clones are listed in Table 2. Clones 14 and 20 reacted with all LPS, giving comparable endpoint titers; both were of the immunoglobulin M (IgM) class. Clones 25 (IgG3) and 27 (IgG3) yielded similar hemolytic titers with R595 and R345 LPS, but did not react with the other chemotypes. None of the antibodies reacted in the passive hemolysis assay with synthetic *E. coli* lipid A or its 1- or 4'-dephospho derivatives. By this reaction pattern, clones 14 and 20 were assumed to recognize a common core structure of all LPS, whereas clones 25 and 27 seemed to react with an epitope expressed only by R595 and R345 LPS. To further support this assumption, clones 20 and 25 were characterized by using synthetic antigens. Similar reaction patterns were obtained with antibodies from culture fluid supernatants which were, however, of much lower activity (titers between 80 and 1,280 [data not shown]). Since these activities were too low for immunodiffusion experiments without being concentrated, further experiments were performed with ascites-derived antibodies.

**Immunodiffusion.** The results obtained by double immunodiffusion experiments using synthetic antigens (copolymerization products of KDO allylglycosides with acrylamide) are shown in Fig. 3. Clone 20 yielded a reaction of identity with poly-α-KDO and poly-$(\alpha\text{-KDO})_2$, but did not react with poly-β-KDO. Clone 25 reacted exclusively with poly-$(\alpha\text{-KDO})_2$ and not with the monosaccharide derivatives in either anomeric configuration.

**Passive hemolysis inhibition.** The two clones were further characterized by inhibition studies. Sheep erythrocytes (SRBC) were sensitized with various amounts of either R595 LPS or the synthetic compound α-KDO-$(GlcNhm)_2$, respectively, and tested with the corresponding antibodies. The optimal sensitization rates for R595 LPS (tested with clone 25) and for α-KDO-$(GlcNhm)_2$ (tested with clone 20) were 4 and 8 μg, respectively. Antibodies were then diluted to yield three hemolytic units of antibody with optimally coated SRBC. These antigen-antibody systems were inhibited by preincubation of the antibody with synthetic antigens and with different rough-form LPS (Table 3). Among the synthetic antigens, only the poly-$(\alpha\text{-KDO})_2$ inhibited the hemolytic system of Re LPS clone 25, yielding an inhibition value of 16 ng. Among the LPS, R595 and R345 LPS were effective inhibitors (inhibition values of 16 and 63 ng, respectively), whereas LPS from other chemotypes were not active (inhibition values between 500 and >1,000 ng). Clone 20 was inhibited by synthetic antigens containing KDO in α linkage, whereas the β-linked KDO was not active in amounts of up to 100 μg. All LPS were inhibitors of this system, with inhibition values ranging from 0.4 to 12.5 μg.

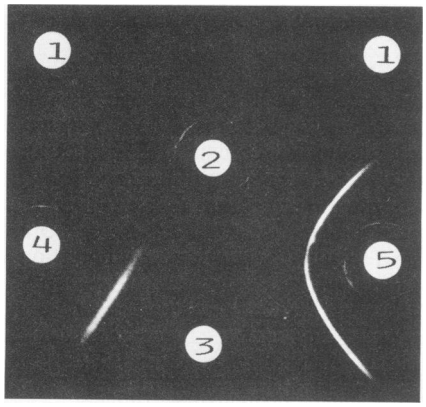


FIG. 3. Reaction patterns of monoclonal antibodies with synthetic KDO antigens in double immunodiffusion experiments. Wells (15 μl) contained 4 μg of either poly-β-KDO (1) or poly-α-KDO (2) or 1 μg of poly-$(\alpha\text{-KDO})_2$ (3), and monoclonal antibody (diluted 1:3 in barbital-glycin-Tris buffer) clones 25 (4) and 20 (5). Diffusion was allowed to proceed at room temperature for 24 h in 1% agarose in barbital-glycin-Tris buffer supplemented with saline (150 mM).

TABLE 3. Passive hemolysis inhibition of monoclonal antibodies with synthetic KDO antigens and LPS from *S. minnesota* rough mutants

| Inhibitor | Inhibition value (ng) obtained in hemolytic system: | | Amt of KDO (mol/2 mol of GlcN)[a] |
|---|---|---|---|
| | Re LPS clone 25 | α-KDO-$(GlcNhm)_2$ clone 20 | |
| Synthetic antigen | | | |
| Poly-α-KDO | >1,000 | 0.2 | |
| Poly-β-KDO | >1,000 | >100 | |
| Poly-$(\alpha\text{-KDO})_2$ | 16 | 0.05 | |
| *S. minnesota* LPS | | | |
| R595 (Re) | 16 | 0.4 | 2.1 |
| R4 ($Rd_2$) | 500 | 6.3 | 2.4 |
| R7 ($Rd_1$) | >1,000 | 12.5 | 2.2 |
| R5 (Rc) | >1,000 | 12.5 | 2.3 |
| R345 ($Rb_2$) | 63 | 1.6 | 3.0 |

[a] The release of KDO was optimized as previously described (2) and calculated per mole of lipid A containing 2 mol of D-glucosamine (GlcN).

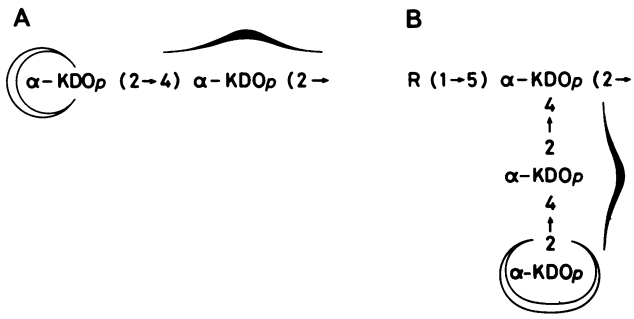


FIG. 4. Schematic representation of the epitope specificities of monoclonal antibodies recognizing KDO structures in the inner core region of enterobacterial rough-form LPS. Open symbols indicate the specificity of clone 20 reacting with an α-linked terminal KDO residue. Closed symbols illustrate the specificity of clone 25 directed against an α-2.4-linked KDO disaccharide. The KDO disaccharide represents the complete core oligosaccharide in Re LPS (A), whereas it is present as a side chain in LPS other than Re. In $Rb_2$ LPS (strain R345), this substitution is quantitative (B), whereas other chemotypes contain it in nonstoichiometric amounts (cf. Fig. 1). R, Core oligosaccharide.

## DISCUSSION

From a set of mouse monoclonal antibodies against the core region of the Re mutant R595 of *S. minnesota*, two clones were selected for their reaction patterns with LPS from different rough mutants of the same species. In the passive hemolysis test, one antibody (clone 20) was reactive with all LPS, whereas another (clone 25) reacted exclusively with Re and $Rb_2$ LPS from *S. minnesota* R595 and R345, respectively. Neither antibody reacted with the lipid A moiety. It was likely that clone 20 reacted with a terminal KDO residue which was present in all LPS tested (3, 4), whereas the reactivity of clone 25 raised the question as to which structure was shared by these two LPS and was absent in the others. From our earlier work on the inner core region of enterobacterial LPS, we know that Re type LPS contains only two pyranosidic KDO residues in α linkage (5, 6), whereas in LPS from mutants other than Re, one KDO is substituted in position 5 by heptose (4) and in position 4 by an α-linked KDO disaccharide (Fig. 1) (35). This KDO disaccharide is sometimes present in nonstoichiometric amounts in most LPS; it is, however, constantly and quantitatively present in $Rb_2$ LPS from *S. minnesota* R345 (2). Therefore, we hypothesized that clone 25 reacts specifically with an α-2,4-linked KDO disaccharide, since it represents the complete core oligosaccharide in Re mutants. The same structural element is present in $Rb_2$ LPS of *S. minnesota* R345 as a side chain, substituting KDO I (cf. Fig. 1) of the main polysaccharide chain. (The designation of the chemotype [$Rb_2$] must not be confused with that of the strain [R345], since we do not yet know whether all mutants of the chemotype $Rb_2$ exhibit this property.)

To prove this hypothesis about the epitope specificities, synthetic antigens were prepared which contained KDO or KDO disaccharide in defined linkages representing common structures of the inner core region (5). Copolymerization products of KDO allylglycosides with acrylamide (10) yielded polyvalent high-molecular-weight antigens which were suitable for immunodiffusion experiments and for inhibition studies, since unlike LPS, they exhibited no hydrophobic properties, which excluded artifacts due to the formation of micelles or aggregates. However, SRBC could not be coated with these synthetic compounds. Therefore, in another synthetic approach, KDO was linked to a part structure of natural lipid A, namely the *N*-acylated D-glucosamine disaccharide backbone (Fig. 2). This compound readily sensitized SRBC, which could be used in the passive hemolysis assay.

By using these synthetic compounds as antigens, the reaction patterns of clones 20 and 25 could be interpreted unequivocally. Clone 20 was specific for a terminal α-linked KDO residue, since (i) it gave a reaction of identity in double immunodiffusion experiments with poly-α-KDO and poly-$(\alpha\text{-KDO})_2$ but not with poly-β-KDO, (ii) it reacted in the passive hemolysis assay with the synthetic compound α-KDO-$(GlcNhm)_2$ and with all LPS to a comparable extent, and (iii) its reaction with the KDO monosaccharide derivative α-KDO-$(GlcNhm)_2$ could be inhibited with all synthetic compounds containing α-linked KDO and with all LPS. Thus, clone 20 was a KDO monosaccharide-specific antibody which, in addition, recognized the α-anomeric configuration.

Clone 25 was identified as an antibody specific for an α-2,4-linked KDO disaccharide, since (i) it reacted only with synthetic poly-$(\alpha\text{-KDO})_2$ in immunodiffusion experiments, (ii) it lysed only SRBC coated with either R595 (Re) or R345 ($Rb_2$) LPS, and (iii) its reaction with Re LPS was exclusively inhibited with poly-$(\alpha\text{-KDO})_2$ and with R595 and R345 LPS. The epitope specificities of these two clones are illustrated schematically in Fig. 4.

These data show that the reaction patterns of monoclonal antibodies with LPS (the same holds true for other natural antigens exhibiting intrinsic heterogeneity) may be misinterpreted or may not be understood at all if the antigen is not defined on a molecular level. Only with knowledge of the structure of the inner core region of LPS was it possible to postulate the hypothesis, and without the availability of synthetic compounds, we would have been unable to verify it.

## ACKNOWLEDGMENTS

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