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Identification of a Cross-reactive Epitope Widely Present in Lipopolysaccharide from Enterobacteria and Recognized by the Cross-protective Monoclonal Antibody WN1 222-5*

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Septic shock due to infections with Gram-negative bacteria is a severe disease with a high mortality rate. We report the identification of the antigenic determinants of an epitope that is present in enterobacterial lipopolysaccharide (LPS) and recognized by a cross-reactive monoclonal antibody (mAb WN1 222-5) regarded as a potential means of treatment. Using whole LPS and a panel of neoglycoconjugates containing purified LPS oligosaccharides obtained from *Escherichia coli* core types R1, R2, R3, and R4, *Salmonella enterica*, and the mutant strain *E. coli* J-5, we showed that mAb WN1 222-5 binds to the distal part of the inner core region and recognizes the structural element R_1-α-D-Glc*p*-(1→3)-[L-α-D-Hep*p*-(1→7)]-L-α-D-Hep*p* 4P-(1→3)-R_2 (where R_1 represents additional sugars of the outer core and R_2 represents additional sugars of the inner core), which is common to LPS from all *E. coli*, *Salmonella*, and *Shigella*. WN1 222-5 binds poorly to molecules that lack the side chain heptose or lack phosphate at the branched heptose. Also molecules that are substituted with Glc*p*N at the side chain heptose are poorly bound. Thus, the side chain heptose and the 4-phosphate on the branched heptose are main determinants of the epitope. We have determined the binding kinetics and affinities (K_D values) of the monovalent interaction of *E. coli* core oligosaccharides with WN1 222-5 by surface plasmon resonance and isothermal titration microcalorimetry. Affinity constants (K_D values) determined by SPR were in the range of 3.6×10^{-5} to 3.2×10^{-8} M, with the highest affinity being observed for the core oligosaccharide from *E. coli* F576 (R2 core type) and the lowest K_D values for those from *E. coli* J-5. Affinities of *E. coli* R1, R3, and R4 oligosaccharides were 5–10-fold lower, and values from the *E. coli* J-5 mutant were 29-fold lower than the R2 core oligosaccharide. Thus, the outer core sugars had a positive effect on binding.

Lipopolysaccharides (LPS[1]; endotoxin) are major surface-exposed structural components of the outer membrane of Gram-negative bacteria (1), and in enterobacteria they consist of lipid A, core region, and O-antigen in many bacteria (2). The lipid A moiety is responsible for many of the pathological effects observed in septic shock, a serious condition with high mortality rates, especially among hospitalized patients in intensive care units. Septic shock is the result of an uncontrolled systemic activation of the immune system by endotoxins, leading to high levels of proinflammatory cytokines such as tumor necrosis factor-α and interleukin-1. In the fight against septicemia, therapeutic strategies are aimed at the eradication of the bacteria by antibiotics, stabilization of the circulation symptomatically, and the neutralization of endotoxic effects. For the last goal, endotoxin antagonists, antibodies against tumor necrosis factor, interleukin-1 receptor antagonists, and LPS-binding proteins (BPI (bactericidal permeability-increasing protein) and LBP (LPS-binding protein)) have been considered (3–6).

Antisera against the O-antigens of endotoxins protect against homologous bacteria. However, the large number of different O-antigens in enterobacteria, the serotype-restricted specificity of such antisera, and the rapid onset of shock have prevented their introduction into clinical practice. Whereas the chemical structure of the O-antigen is highly variable, the core region and lipid A show only limited structural variability within the enterobacteria. Following the observation that antibodies against the O-antigen are protective against homologous bacteria, the search for LPS antibodies with broad cross-reactivity is a valid concept for the immunotherapy of Gram-negative sepsis. Many investigators attempted the isolation of antibodies that are directed against the conserved regions of LPS (*i.e.* the lipid A and core region (reviewed in Ref. 7)). Such antibodies have been presumed to be cross-reactive and cross-protective against different Gram-negative pathogens. Such a cross-protective effect was described for a polyclonal antiserum by Braude and Douglas (8); however, all subsequently isolated LPS-specific monoclonal antibodies failed to show cross-reactivity *in vitro* and cross-protectivity *in vivo* (7), with the exception of mAb WN1 222-5 (9). This mAb bound to LPS from all tested clinical isolates of *Escherichia coli*, *Salmonella*, and

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[1] The abbreviations used are: LPS, lipopolysaccharide; DQF-COSY, double quantum-filtered correlation spectroscopy; Glc*p*, glucopyranose; Glc*p*N, 2-amino-2-deoxy-glucopyranose, L-α-D-Hep*p*, L-*glycero*-α-D-*manno*-heptopyranose; HMQC, heteronuclear multiple quantum correlation; HPAEC, high performance anion exchange chromatography; ITC, isothermal titration microcalorimetry; Kdo, 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid; SPR, surface plasmon resonance; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

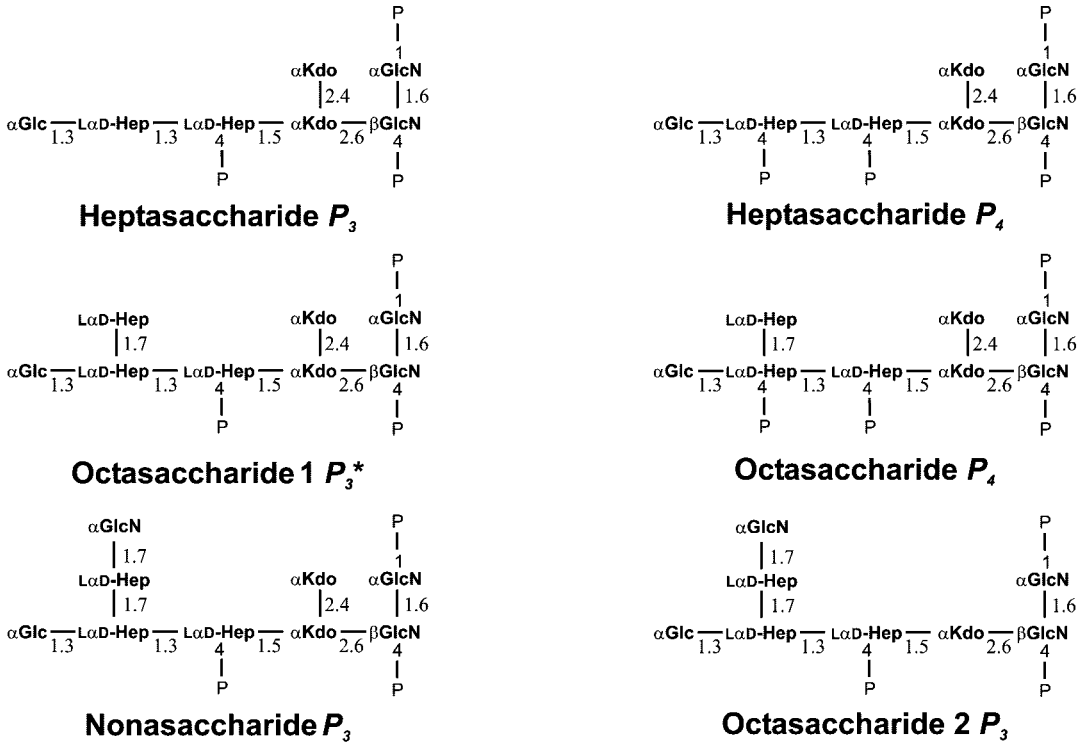

FIG. 1. **Chemical structures of core oligosaccharides obtained after alkaline deacylation of *E. coli* J-5 LPS.** The *E. coli* J-5 mutant is a derivative of *E. coli* O111:B4, which expresses a defective UDP-galactose-4-epimerase (10) and is therefore unable to incorporate galactose into its LPS. The separation of LPS into individual molecular species is so far not possible due to the amphiphilic nature of LPS. After successive de-*O*- and de-*N*-acylation, LPS oligosaccharides can be separated by HPAEC, and the depicted oligosaccharides were obtained from the J-5 mutant. Octasaccharide 1 P_3, marked with an *asterisk*, was only obtained after chemical deamination of LPS before the deacylation and is not naturally present in LPS from *E. coli* J-5.

Shigella in Western blots and ELISA and showed cross-protective effects *in vivo* against the endotoxic activities of LPS (9). The smallest LPS structure bound by WN1 222-5 was found to be present in LPS from *E. coli* J-5. Due to the lack of a functional UDP-galactose-4-epimerase (ΔgalE mutant) (10), this strain is unable to incorporate galactose into its LPS and therefore produces a truncated LPS consisting of several glycoforms (Fig. 1). The cross-reactivity was therefore attributed to a common epitope located in the inner core region of these LPS (9).

LPS preparations are heterogenous and there are no methods available for the separation of acylated LPS into homogeneous compounds due to their amphiphilic nature. For this reason, the exact epitope of WN1 222-5 could not be determined using LPS. We have therefore developed methods for the deacylation of these molecules under conditions that do not cleave glycosidic bonds (11) and the purification of LPS oligosaccharides. These oligosaccharides are then amenable to a detailed structural characterization and conjugation to proteins (12, 13).

For J-5 LPS, five different oligosaccharides, which differ in their carbohydrate structures and phosphate substitution (Fig. 1), were obtained by deacylation under strong alkaline conditions (14). The chemical structures of five different *E. coli* core types (R1 to R4 and K-12) and two chemically distinct core oligosaccharides of *S. enterica* (2, 15–17) are known, and all possess identical inner core structures (Fig. 2). Minor differences in the inner core structures relate to the substitution of the side chain heptose with Glc*p*N and the concomitant lack of phosphate on the branched heptose residue. Major structural differences between the core types are observed in the outer core region. Identical inner core structures have been described for *Shigella* species (2).

Using a panel of highly purified oligosaccharides from *Salmonella enterica* sv. Minnesota (R1), *E. coli* F470 (R1 core-type), *E. coli* F576 (R2 core-type), *E. coli* F653 (R3 core-type), *E. coli* F2513 (R4 core-type), and *E. coli* J-5, shown in Figs. 1 and 2, we have determined the minimal epitope that is recognized by mAb WN1 222-5 by ELISA, ELISA inhibition, isothermal titration microcalorimetry (ITC), and surface plasmon resonance (SPR) and studied the influence of the outer core on the binding of WN1 222-5 to enterobacterial LPS.

EXPERIMENTAL PROCEDURES

Bacteria, Extraction of LPS, and Isolation of Oligosaccharides—*E. coli* F470 (R1 core-type) (15), *E. coli* F576 (R2 core-type) (15), *E. coli* F653 (R3 core type) (16), *E. coli* F2513 (R4 core type) (16), *Salmonella enterica* sv. Minnesota (*Salmonella* R1 core type) (17), and the *E. coli* strain J-5 (ΔgalE mutant) (14) were cultivated, and LPS was extracted from each. Briefly, LPS was isolated by phenol/chloroform/petrolether extraction (18) and de-*O*-acylated by mild hydrazinolysis, followed by de-*N*-acylation under strong alkaline conditions (4 M KOH, 120 °C, 16 h) (11). After 3-fold extraction with chloroform, the mixture of deacylated oligosaccharides was separated by high performance anion exchange chromatography (HPAEC) as reported (14–16). Pure oligosaccharides were then desalted by gel chromatography on Sephadex G-10 in 10 mM NH_4HCO_3, followed by lyophilization.

Deamination of E. coli J-5 LPS—LPS isolated from *E. coli* J-5 was subjected to a deamination reaction as described by Vinogradov *et al.* (19). One ml of acetic acid and 200 mg of $NaNO_2$ were added to 200 mg of LPS in 10 ml of water, and after a 12-h incubation at ambient temperature, the deaminated LPS was collected by centrifugation (4 h, 4 °C, 120,000 × *g*). The precipitate was dissolved in water and dialyzed against deionized water (3× 1 liter, 4 °C) and lyophilized (yield: 149 mg). An aliquot (50 mg) was then de-*O*- and de-*N*-acylated, yielding four oligosaccharides, which were isolated by semipreparative HPAEC and gel filtration as described above (oligosaccharide (OS) 1, 4.2 mg; OS 2, 1.8 mg; OS 3, 2.3 mg; OS 4, 0.8 mg).

Neoglycoconjugates—Neoglycoconjugates of deacylated oligosaccharides were prepared as described (20). Briefly, ligands (2.5 mg) were dissolved in 200 μl of 50 mM carbonate buffer, pH 9.2; glutardialdehyde (25%, electron microscopy grade; Merck) was added (1% final concentration); and the sample was stirred for 4 h at 25 °C under N_2 atmo-

sphere. Excess glutardialdehyde was removed by lyophilization, and the samples were redissolved in 200 μl of water. BSA (2.5 mg) was added from a 10 mg ml^{-1} solution in 50 mM carbonate buffer, pH 9.2, and the mixture was incubated overnight at 25 °C. Finally, 250 μg of $NaBH_4$ was added, and the samples were incubated for 1 h at 4 °C in the dark followed by dialysis against water once and three times against PBS, pH 7.2.

mAb WN1 222-5—The generation and selection of mAb WN1 222-5 has been described in detail previously (9). Stock solutions of affinity purified mAb were kept at −20 °C in aliquots (1 mg ml^{-1}).

ELISA—Binding of mAb WN1 222-5 to the neoglycoconjugates was determined by ELISA. Varying amounts of glycoconjugates were coated onto 96-well microtiter plates (Nunc, Maxisorb) and tested against serial dilutions of antibody. Antibody binding was detected with enzyme-conjugated anti-mouse IgG and substrate and measured photometrically at 405 nM. Experiments were done in quadruplicate, and mean values were calculated. Confidence values did not exceed 10%. Binding of the mAb WN1 222-5 to fully acylated LPS was determined using LPS as a solid phase antigen instead of neoglycoconjugates (21).

For ELISA inhibition, serial dilutions of inhibitor in PBS-Tween 20/casein/BSA (30 μl) were mixed in V-shaped microtiter plates (NUNC) with an equal volume of antibody diluted in the same buffer to give an A_{405} of 1.0 without the addition of inhibitor. After incubation (15 min, 37 °C), 50 μl of the mixture were added to antigen-coated ELISA plates. Further steps were as described above. All measurements were done at least twice in duplicate with confidence values not exceeding 20%.

Surface Plasmon Resonance—Analyses were performed with a BIACORE 3000 instrument (Biacore, Inc.). WN1 222-5 was immobilized on a CM5 sensor chip (Biacore) at a surface density of ~20,000 RU using the amine coupling kit from Biacore. Analyses were carried out at 25 °C in 10 mM HEPES, pH 7.4, containing 3 mM EDTA, 0.005% P-20, and 150 mM or 300 mM NaCl. Surface regeneration was not necessary. Data were evaluated using the BIAevaluation 3.0 software (Biacore).

Isothermal Titration Microcalorimetry—Microcalorimetric experiments were performed on an MCS isothermal titration calorimeter (Microcal Inc., Northampton, MA). mAb WN1 222-5 was dialyzed against PBS, pH 7.2, and the concentration was determined by UV measurement (1 mg ml^{-1} = A_{280} of 1.35). The mAb concentration was adjusted to 7.55 μM, assuming a molecular mass of 150 kDa, and the microcalorimeter cell was filled with the antibody solution (volume = 1.3 ml). Purified and desalted deacylated LPS oligosaccharide were dissolved at a concentration of 0.35 mM in dialysis buffer and loaded into the syringe of the microcalorimeter. Both solutions were thoroughly degassed prior to loading. After temperature equilibration, the ligand was injected into the cell in 5-μl portions, and the evolved heat was measured with the first injection not considered for data analysis. A total of 20 injections were performed with 5-min equilibration times between injections. Data were corrected for heat of dilution by measuring the same number of buffer injections and subtraction from the sample data set. Dissociation constants were determined using the MicroCal Origin version 2.9 analysis software and the model of 1 set of binding sites. The antibody concentration in the cell was corrected after the curve fitting as described in the ITC Data Analysis in the Origin Version 2.9 manual provided by the manufacturer.

Nuclear Magnetic Resonance—^{1}H (600.12 MHz), ^{13}C (150.13 MHz), and ^{31}P (242.13 MHz) NMR spectra were recorded with a Bruker DRX Avance spectrometer with a 4-mg sample in 0.5 ml of D_2O. Acetone (2.225 ppm) (^{1}H) and dioxane (67.4 ppm) (^{13}C) served as references. All spectra were run at a temperature of 300 K. One-dimensional ^{1}H, ^{13}C, and ^{31}P and two-dimensional homonuclear $^{1}H,^{1}H$ (DQF-COSY, NOESY, TOCSY), heteronuclear $^{1}H,^{13}C$, and $^{1}H,^{31}P$ NMR correlation spectra (HMQC) were recorded using Bruker standard pulse programs and analyzed with Bruker Xwinnmr software.

RESULTS

Oligosaccharide Preparation—Deacylation of LPS from *E. coli* strains F470 (15), F576 (15), F563 (16), and F2513 (16), purification of oligosaccharides, and characterization of the chemical structures has been described in the respective publications. *S. enterica* sv. Minnesota LPS was isolated as described (17) and treated according to the same procedure as the other LPS. The chemical structures are depicted in Fig. 2. Deacylation of LPS from *E. coli* J-5 yielded five oligosaccharides (Fig. 1), which differed in their carbohydrate structure and phosphate substitution (14). We have prepared an additional oligosaccharide from *E. coli* J-5 by deamination of LPS and subsequent deacylation under strong alkaline conditions that lacked phosphate at the branched heptose and was devoid of Glc*p*N on the side chain heptose. *E. coli* J-5 does not naturally produce such an oligosaccharide. Analysis by analytical HPAEC (Fig. 3*A*) revealed retention times of 25.7, 27.7, 35.0, and 37.3 min for oligosaccharides 1–4, respectively. Oligosaccharides 2–4 possessed identical retention times to those of deacylated *E. coli* J-5 LPS without deamination (Fig. 3*B*). From previous studies, it was known that these are heptasaccharide P_3 (Fig. 3, *OS 2*), octasaccharide P_4 (*OS 3*), and heptasaccharide P_4 (*OS 4*). This was confirmed by ^{1}H NMR spectroscopy of the purified oligosaccharides. There was no signal at a retention time of nonasaccharide P_3 (18.7 min, OS 5) after deamination, and a new peak appeared at 25.7 min (OS 1). A ^{1}H NMR spectrum of the latter compound (Fig. 4) contained six signals of anomeric protons and two pairs of signals of deoxyprotons, indicating the presence of two Kdo residues. Comparison with the ^{1}H NMR spectrum of nonasaccharide P_3 of deacylated *E. coli* J-5 LPS indicated that the side chain Glc*p*N was missing. This was finally proven by the assignment of all ^{1}H and ^{13}C NMR chemical shifts (Table I), determination of coupling constants by $^{1}H,^{1}H$ DQF-COSY, and nuclear Overhauser effect NMR experiments. Thus, this oligosaccharide had the same carbohydrate structure as octasaccharide P_4 (Fig. 1). However, the upfield resonance frequencies of proton H-4 and carbon C-4 of the second heptose residue indicated that this position was not substituted with phosphate (Fig. 1). The ^{31}P NMR spectrum accordingly contained only three resonances (Fig. 5) with chemical shifts of −1.41, 0.54, and 1.60 ppm. Therefore, this oligosaccharide, designated octasaccharide 1 P_3, contained only three phosphate residues, and a $^{1}H,^{31}P$ HMQC-COSY NMR experiment proved that the phosphate at the 4-position of the second heptose (residue *F* in Fig. 4) was absent as in nonasaccharide P_3.

ELISA with LPS and Complete Core Structures—It was previously shown that WN1 222-5 binds to whole cells of *E. coli* and *S. enterica* and to the LPS of these bacteria in Western blots and passive immunohemolysis (9). The minimal LPS structure bound by WN1 222-5 was the LPS of the rough mutant strain *E. coli* J-5. To verify this reactivity by ELISA, we first immobilized LPS of *E. coli* core types R1 to R4 and *S. enterica* sv. Minnesota on microtiter plates and investigated their reactivity with mAb WN1 222-5 (Fig. 6*A*). The antibody reacted with all of these LPS. We then investigated whether the lipid A was important for the binding and studied the inhibitory activities of deacylated LPS oligosaccharides by ELISA inhibition. When LPS was treated with mild acid, the mixture of *E. coli* R3 deacylated LPS oligosaccharides did not show any inhibitory activity up to the concentration tested (5 μg/well; see Table III). On the contrary, oligosaccharides from the same LPS obtained by deacylation under alkaline conditions, which retained the lipid A backbone sugars and the side chain Kdo substitution, possessed inhibitory activity (50% inhibition at 20 ng/well). Therefore, fatty acids did not influence the binding and were not part of the WN1 222-5 epitope. As can be seen in Fig. 6*B*, mAb WN1 222-5 bound to all tested BSA-neoglycoconjugates of *E. coli* LPS obtained after alkaline deacylation to the same extent as to LPS. All further experiments were therefore done with oligosaccharides obtained after alkaline deacylation.

It is known that LPS from *E. coli* F470 and F653 (R1 and R3 core, respectively) contain core structures in which the side chain heptose is substituted by Glc*p*N via an α-1→7 linkage (15, 16). Concomitantly, the phosphate at the second heptose is missing in these molecules (16). In order to investigate the

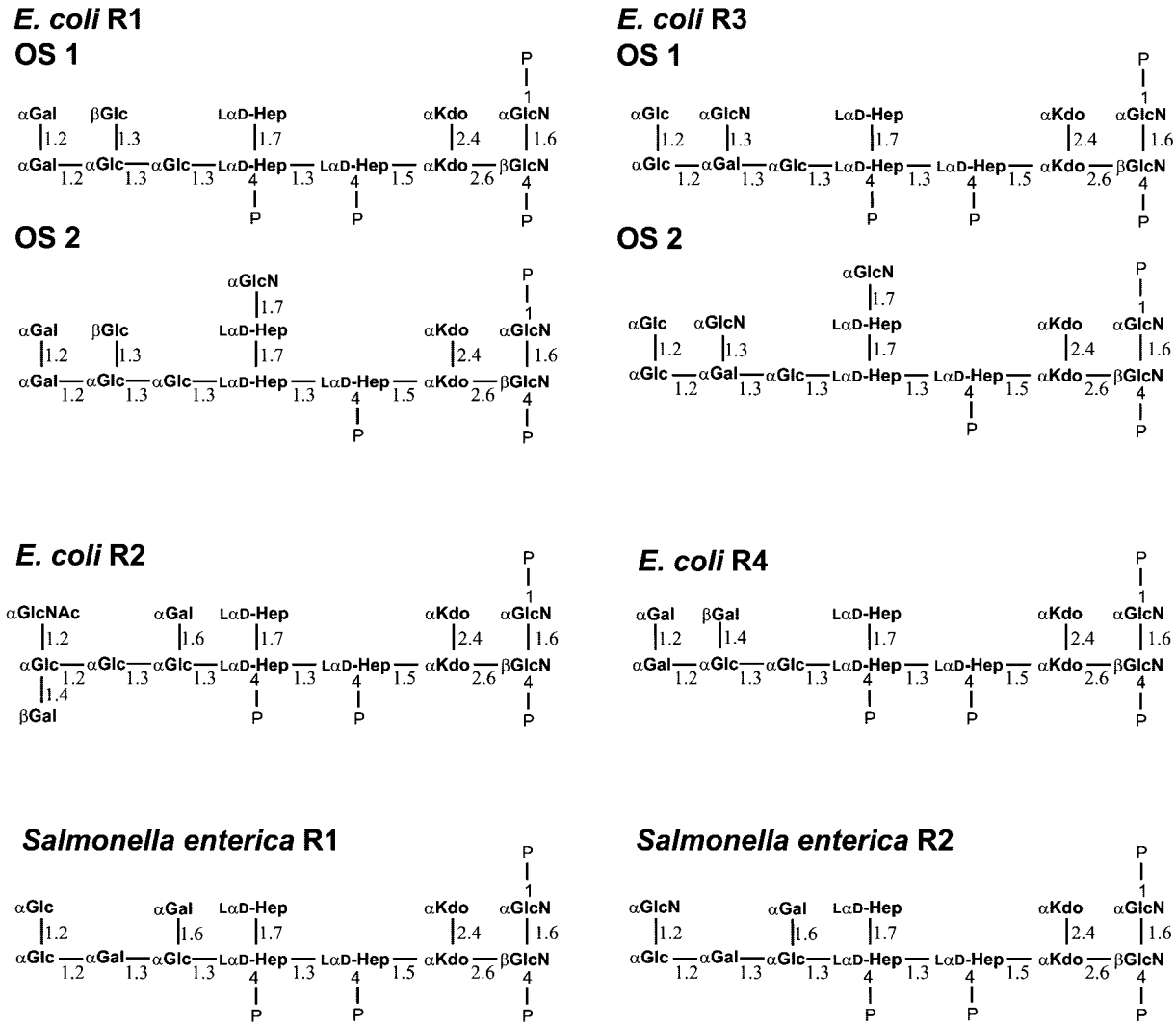

FIG. 2. **Chemical structures of core-oligosaccharides obtained after alkaline deacylation of *E. coli* and *Salmonella* LPS.** Separation of deacylated LPS from *E. coli* strains F470 (R1), F576 (R2), F653 (R3), and F2513 (R4), *S. enterica* sv. Minnesota (R1), and *S. enterica* sv. (R2) by HPAEC identified the depicted oligosaccharides as major components (15–17). Whereas LPS from *E. coli* F470 and F653 contain two oligosaccharides (OS 1 and 2) differing in the side chain heptose substitution with Glc*p*N, this modification is absent in LPS of the other strains.

influence of these structural variations on the binding of mAb WN1 222-5, we have purified these oligosaccharides and prepared neoglycoconjugates thereof. These did not bind to WN1 222-5 in ELISA (data not shown), indicating that either the lack of phosphate substitution at this position, the substitution of the side chain heptose by Glc*p*N, or both influence binding by WN1 222-5. These results were confirmed by ELISA inhibition (see Table III), where the complete core oligosaccharide 2 of *E. coli* F653 (R3 core containing the Glc*p*N side chain substitution) was unable to inhibit the interaction between WN1 222-5 and the R3 core oligosaccharide 1 (without Glc*p*N in the core). It was found that the core oligosaccharide of *E. coli* F576 (R2 core) was the best inhibitor in this system.

ELISA with J-5 Core Oligosaccharides—Aiming at the identification of the minimal epitope required for the binding of WN1 222-5, we have conjugated each of the oligosaccharides obtained from *E. coli* J-5 LPS and the newly prepared octasaccharide 1 P_3 to BSA using glutardialdehyde coupling (20). The octasaccharide 1 P_3 conjugate was included to elucidate the importance of phosphate substitution and side chain heptose substitution with Glc*p*N for antigen binding by WN1 222-5. The neoglycoconjugates were immobilized on ELISA plates, and the reactivity was tested with mAb WN1 222-5. As shown in Fig. 7, mAb WN1 222-5 did not bind to nonasaccharide P_3 and heptasaccharide P_3, whereas intermediate binding to heptasaccharide P_4 and octasaccharide 1 P_3 was observed. The highest affinity was observed for the interaction of WN1 222-5 and octasaccharide P_4.

In ELISA inhibition (see Table III), nonasaccharide P_3 and heptasaccharide P_3 were both unable to inhibit the interaction between WN1 222-5 and the R3 core-oligosaccharide 1 (without Glc*p*N in the core), whereas heptasaccharide P_4 and octasaccharide 1 P_3 showed inhibitory activity, yielding 50% inhibition values at concentrations of 15 and 3.6 μM, respectively. Octasaccharide P_4 was the best inhibitor among the J-5 oligosaccharides and showed an inhibitory activity comparable with the homologous R3 oligosaccharide 1.

Affinity and Kinetic Constants Determined by SPR and Microcalorimetry—In order to gain a deeper insight into the kinetics and affinities of the binding, we performed SPR and isothermal titration microcalorimetry analyses of the binding of core oligosaccharides to WN1 222-5. For SPR, WN1 222-5 was immobilized, and purified oligosaccharides were used as analytes at different concentrations. Although three of the J-5 oligosaccharides are known to bind to WN1 222-5 (Fig. 7), interactions were not observed by SPR in buffer containing the standard NaCl concentration of 150 mM. Binding of the J-5 oligosaccharides was observed only when the NaCl concentra-

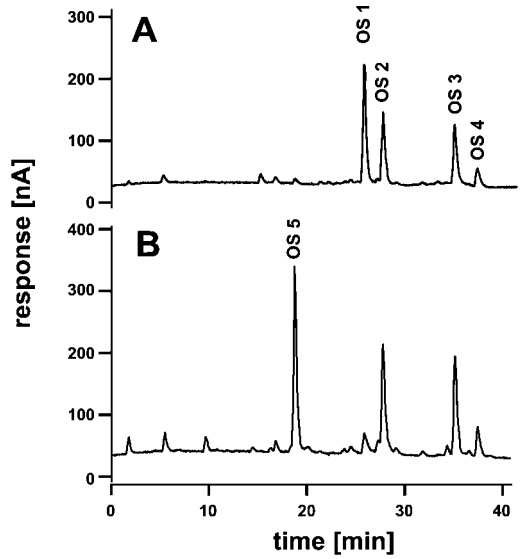

FIG. 3. **Comparison by HPAEC of *E. coli* J-5 LPS core oligosaccharides that were obtained after deacylation of deaminated (*A*) and native (*B*) LPS.** Analytical HPAEC was carried out on a column of CarboPak PA1 (4 × 250 mM; Dionex) using the eluents H_2O (*A*) and 1 M NaOAc (*B*) and a gradient from 1 to 99% of eluent B over 80 min. The run was monitored by pulsed amperometric detection after the postcolumn addition of NaOH. OS 2–4 correspond to heptasaccharide P_3, octasaccharide P_4, and heptasaccharide P_4, respectively, of the structures shown in Fig. 1. The deamination converted OS 5 (nonasaccharide P_3) into OS 1 (octasaccharide 1 P_3), which was devoid of the side chain Glc*p*N substitution and did not contain phosphate on the middle L-α-D-Hep.

tion was increased to 300 mM. The complete core oligosaccharides bound to WN1 222-5 at both salt concentrations but gave different K_D values at different salt concentrations. Surprisingly, the affinities were higher at 300 mM NaCl, and this was due to faster on-rates; the off-rates remained essentially unchanged. The affinities were 0.6–25-fold higher at 300 mM NaCl in comparison with those obtained at 150 mM NaCl (Table II). At 150 mM NaCl, measurements were only possible at a low flow rate of 5 μl/min due to matrix effects. Measurements at 300 mM NaCl allowed higher flow rates without changes of the matrix, reducing the risk of mass transport limitations. Global fitting of the data collected at both salt concentrations deviated only slightly from a 1:1 interaction model (Fig. 8). With most of the oligosaccharides, equilibrium data were collected for derivation of K_D values by Scatchard analysis (Fig. 9; Table II).

In view of the discrepancies in the SPR results, we determined the K_D value of the interaction of WN1 222-5 with the R4 core oligosaccharide by isothermal titration microcalorimetry at both salt concentrations (Fig. 10). The K_D values were determined to be 48 nM at 150 mM NaCl and 74 nM at 300 mM NaCl (Table II), thus indicating stronger binding than determined by SPR (K_D = 290 nM by SPR). In order to verify that the relative affinities measured by microcalorimetry were the same as by SPR, we also determined the binding of the *E. coli* R2 and R3 core oligosaccharides; the relative affinities were the same as determined by SPR at 300 mM NaCl. The molecule that bound most strongly in microcalorimetry and SPR measurements was the *E. coli* R2 core oligosaccharide; the K_D values determined by SPR and microcalorimetry were 32 nM (calculated based on $k_{on} = 1.4 \times 10^5\ m^{-1}\ s^{-1}$ and $k_{off} = 4.6 \times 10^{-3}\ s^{-1}$) and 5.5 nM, respectively.

At 300 mM NaCl, the K_D values determined by SPR for the *E. coli* J-5 oligosaccharides confirmed the results obtained by ELISA. Thus, octasaccharide P_4 showed the highest affinity with a K_D of 950 nM. Nonasaccharide P_3 and heptasaccharide P_3 bound only poorly, with K_D values of 36 and 33 μM, respectively. An intermediate affinity was determined for octasaccharide 1 P_3 with a K_D of 2.8 μM.

Comparing the SPR K_D values on a relative basis with the *E. coli* R3 core oligosaccharide revealed that the core oligosaccharides with an outer core all bound more tightly to WN1 222-5 than the smaller core oligosaccharides of the mutant *E. coli* J-5. Whereas octasaccharide P_4 was the best binder among the latter, it showed only a relative affinity of 38% in comparison with the complete core oligosaccharide of the homologous chemotype R3. By far the best binding oligosaccharide was the complete core oligosaccharide of the R2 chemotype with an 11-fold higher affinity than the *E. coli* R3 core oligosaccharide.

DISCUSSION

The concept of treatment of septic shock in humans by active or passive vaccination with cross-protective antibodies was developed after a report by Braude and Douglas, who showed that an antiserum in rabbits was cross-protective against the local Shwartzman reaction induced by heterologous bacteria (8). Since then, a great effort was undertaken to isolate cross-reactive antibodies against LPS on the assumption that they would be cross-protective (reviewed in Ref. 7). To date, WN1 222-5 is the only antibody that has been demonstrated in different experimental settings to be cross-reactive against whole bacteria and isolated LPS from a large number of clinical specimens of *E. coli*, *S. enterica*, and *Shigella* (9).

A detailed epitope analysis requires the separation of individual LPS components, which is so far not possible for acylated LPS due to their amphiphilic nature. For this reason, LPS is commonly deacylated, and this can be achieved in two ways. Mild acid treatment of LPS can be applied for the cleavage of the acid-labile Kdo-lipid A linkage. The insoluble lipid A can then be removed by centrifugation, and LPS oligosaccharides are obtained. However, oligosaccharides prepared in this way lose the lipid A backbone sugars and also the side chain Kdo residue. Such oligosaccharides did not bind to WN1 222-5 in ELISA inhibition (Table III). By contrast, oligosaccharides that were obtained by deacylation under alkaline conditions and still contained the intact lipid A backbone and the Kdo region did bind to WN1 222-5 and thus revealed that fatty acids are not part of the epitope. Therefore, it was possible to use such oligosaccharides after purification in SPR, ITC microcalorimetry, and inhibition test systems and as neoglycoconjugates in ELISA. The analysis in these binding assays revealed that the epitope bound by WN1 222-5 lies in the junction between the inner and the outer core of these LPS and is composed of the structural element α-D-Glc*p*-(1→3)-[L-α-D-Hep*p*-(1→7)]-L-α-D-Hep*p* 4P-(1→, which is common to LPS from *E. coli*, *Salmonella*, and *Shigella* (2). LPS molecules that are devoid of the side chain heptose, as in the heptasaccharide P_4 of *E. coli* J-5, or the phosphate at the second heptose, as in octasaccharide 1 P_3, have affinities that are 21- and 3-fold lower, respectively, than that observed for the complete epitope. Molecules that lack phosphate at the branched heptose and the side chain heptose, such as heptasaccharide P_3, or in which the side chain heptose is masked by Glc*p*N substitution, such as nonasaccharide P_3, are not recognized by WN1 222-5. From these data, it is evident that the most important contributions to the binding come from the side chain heptose and the phosphate at the 4-position of the branched heptose. Furthermore, either the lipid A backbone, the side chain Kdo, or both are necessary for WN1 222-5 reactivity. The contribution of the Glc*p*-residue adjacent to the branched heptose at position 3 cannot be evaluated, because rough mutants that cannot incorporate Glc at this position are also devoid of the side chain heptose (22). We have unsuccessfully attempted to remove the terminal Glc of octasaccharide P_4 using α-glucosidases.

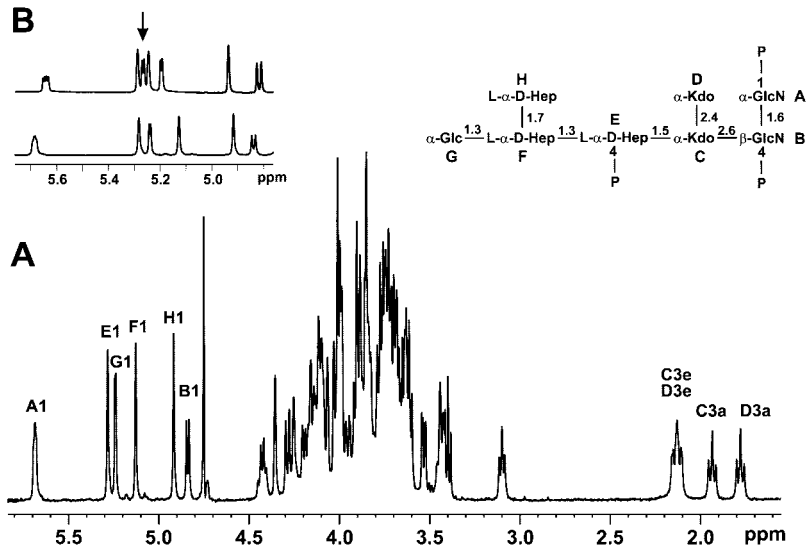

FIG. 4. *A*, ^{1}H NMR spectrum of octasaccharide 1 P_3 obtained after deamination and deacylation of *E. coli* J-5 LPS. Six signals of anomeric protons originating from anomeric protons as indicated (labeling of residues as in the structure depicted) and two pairs of signals from deoxyprotons of Kdo residues (residues *C* and *D*) identified the isolated oligosaccharide as an octasaccharide. *B*, the region of anomeric protons of nonasaccharide P_3 (*top*) in comparison with octasaccharide 1 P_3 (*bottom*) did contain a signal of an additional Glc*p*N residue (marked by an *arrow*), which was absent in octasaccharide P_3.

TABLE I
^{1}H and ^{13}C NMR chemical shift data for octasaccharide 1 P_3 obtained by deamination and deacylation of E. coli J-5 LPS

Residue	Chemical shift											
	H-1 C-1	H-2 C-2	H-3ax C-3	H-3eq	H-4 C-4	H-5 C-5	H-6a C-6	H-6b	H-7a C-7	H-7b	H-8a C-8	H-8b
							PPM					
A →6-α-GlcN 1*P*	5.685	3.430	3.906		3.612	4.116	3.768	4.290				
	91.66	54.74	69.84		69.92	72.96	70.03					
B →6-β-GlcN 4*P*	4.840	3.098	3.871		3.815	3.743	3.453	3.675				
	99.72	55.97	72.33		74.82	74.29	62.78					
C →4,5-α-Kdo			1.934	2.125	4.126	4.258	3.696		3.844		3.897	3.605
	ND[a]	ND	34.78		70.90	70.03	72.69		70.03		64.14	
D α-Kdo			1.777	2.125	4.107	4.029	3.649		3.993		3.947	3.746
	ND	ND	35.28		66.34	67.00	72.69		70.43		63.63	
E →3-α-Hep 4*P*	5.282	4.064	4.157		4.427	4.204	4.096		3.672	3.731		
	99.55	71.27	77.28		70.66	72.80	69.48		63.43			
F →3,7-α-Hep	5.127	4.355	4.016		4.018	3.692	4.169		3.72-3.75	3.72-3.75		
	102.70	69.95	78.97		66.10	72.67	68.64		70.76			
G →3-α-Glc	5.240	3.531	3.780		3.397	3.847	3.903	3.746				
	100.77	72.21	73.34		70.10	72.55	60.88					
H →7α-Hep	4.916	3.985	3.855		3.860	3.639	4.010		3.672	3.725		
	101.43	70.41	71.00		66.58	71.98	69.40		63.51			

[a] ND, not determined.

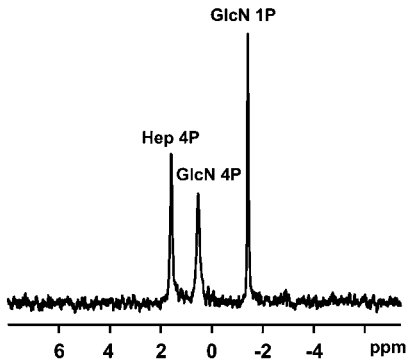

FIG. 5. **^{31}P NMR spectrum of octasaccharide 1 P_3 obtained after deamination and deacylation of *E. coli* J-5 LPS.** Three signals of ^{31}P nuclei proved the presence of three phosphate groups that were substituting positions 1 and 4′ of the lipid A Glc*p*N residues and position 4 of L-α-D-Hep (residue *E*; for structure see *inset* in Fig. 4).

The presence of the outer core sugar residues does not inhibit the reactivity but on the contrary has a positive influence on the affinities. Thus, WN1 222-5 possesses the highest affinity for deacylated LPS from *E. coli* F576 (R2 core) with an affinity constant (K_D) of 3.2×10^{-8} M determined by SPR. The interaction with LPS of other *E. coli* core types was reduced about 5-fold for the R1 and 10-fold for the R3 and R4 core oligosaccharide. In comparison with octasaccharide P_4 (K_D = 9.5×10^{-7} m), which was the best binding molecule derived from *E. coli* J-5, the affinities of molecules possessing an outer core were ~3–5-fold higher and even 29-fold higher for the *E. coli* R2 core oligosaccharide. The reason for the higher affinity of the R2 core oligosaccharide may be the α-1→6-Gal*p*-substitution at the Glc*p* residue, which is attached to the branched heptose (Fig. 2). Whether this residue is involved in a direct interaction or has an influence on the conformation of the neighboring sugars cannot be determined at this stage.

Why oligosaccharides without the lipid A backbone and side chain Kdo are not bound by WN1 222-5 must remain undetermined at this stage. One explanation could be that a conformational epitope is formed by sugars of the lipid A, Kdo residues, and the heptoses, and parts of these sugars are involved in a direct interaction with the antibody. We (16) and others (15) have recently described NMR data that indicate a conformational proximity of the side chain heptose and the inner Kdo, which may explain the dependence of binding on the intact inner core. A direct interaction of these sugars with WN1 222-5 in the combining site, however, seems unlikely, in the light of results obtained by crystallization of antibodies in complex with carbohydrate antigens (23, 24). In such complexes, it has been observed that even of a polysaccharide not more than a

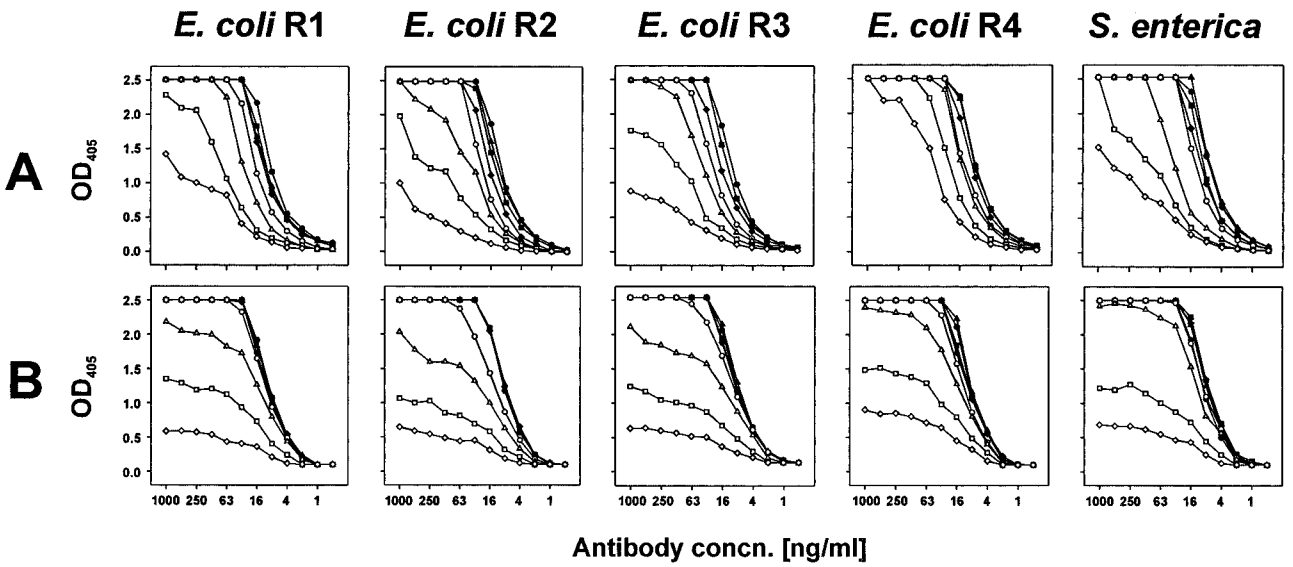

FIG. 6. **Interaction of WN1 222-5 with LPS and core oligosaccharides conjugated to BSA.** ELISA binding curves of the interaction of WN1 222-5 at different concentrations with LPS (*A*) and core oligosaccharides (for structures, see Fig. 1) conjugated to BSA (*B*) of *E. coli* F470 (R1), F576 (R2), F653 (R3), 2513 (R4), and *S. enterica* sv. Minnesota (R1) at a concentration of immobilized ligand of 50 pmol (●), 25 pmol (▲), 12.5 pmol (■), 6.25 pmol (♦), 3.125 pmol (○), 1.56 pmol (△), 0.79 pmol (□), and 0.39 pmol (◇).

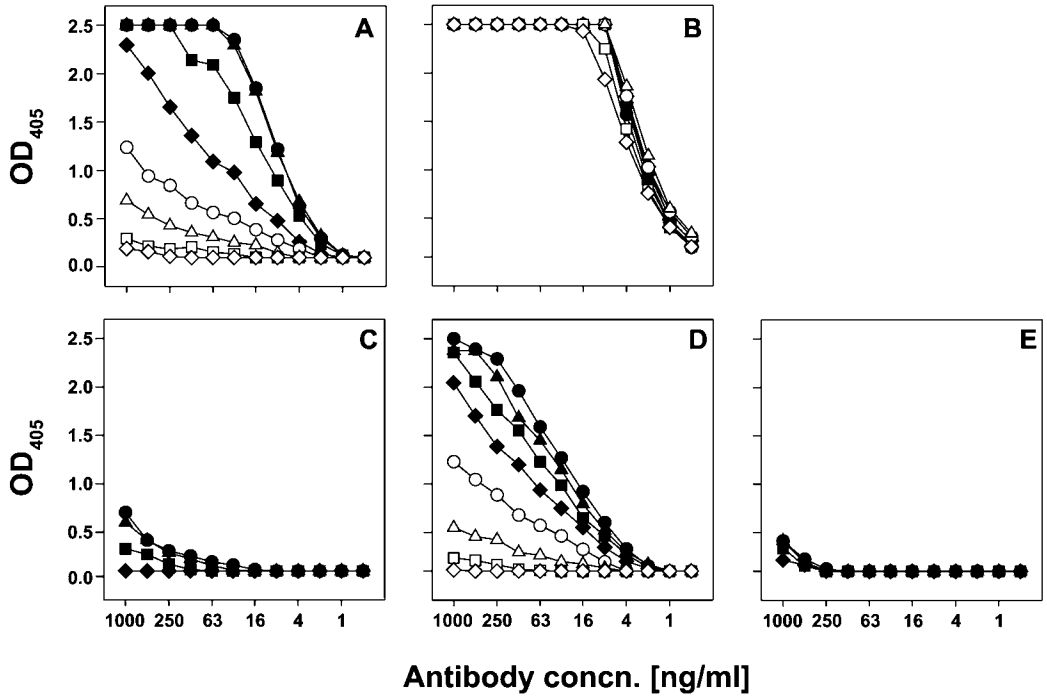

FIG. 7. **Interaction of WN1 222-5 with core oligosaccharides of *E. coli* J-5 conjugated to BSA.** ELISA binding curves of the interaction of WN1 222-5 at different concentrations with neoglycoconjugates of *E. coli* J-5 core oligosaccharides (for structures, see Fig. 2). *A–E*, heptasaccharide P_4, octasaccharide P_4, heptasaccharide P_3, octasaccharide 1 P_3, and nonasaccharide P_3, respectively, coated at ligand concentrations of 50 pmol (●), 25 pmol (▲), 12.5 pmol (■), 6.25 pmol (♦), 3.125 pmol (○), 1.56 pmol (△), 0.79 pmol (□), and 0.39 pmol (◇).

trisaccharide epitope is accommodated in the antibody combining site, and a single sugar is buried in a deep pocket involved in tight interactions with the antibody. It seems therefore unlikely that the distal sugars of the lipid A backbone are involved in the interaction. Molecular modeling calculations indicated a compact conformation of the Kdo residues and the lipid A based on ionic interactions between the lipid A phosphates and carboxylic groups of the Kdo (25). It may therefore be that the removal of the lipid A and/or the side chain Kdo translates into conformational changes of other core sugars further away. In this case, the Kdo and lipid A sugar residues would not be directly involved in the interaction but would influence the binding to WN1 222-5. Experimental evidence may come from the observation that NMR chemical shift values of the anomeric protons of the heptoses significantly change in octasaccharide 2 P_3 of *E. coli* J-5, where the side chain Kdo is missing (14).

The dramatic effect of ionic strength on the affinities of the WN1 222-5 interaction with various oligosaccharides as determined by SPR highlights the importance of not relying on a single technique for analysis of molecular interactions. It is presumed that the salt effect observed with the SPR analyses relates to the carboxylated dextran matrix on CM5 sensor chips. The negatively charged matrix must in some way inter-

TABLE II

Determination of binding constants of the interaction of enterobacterial LPS oligosaccharides with WN1 222–5 at different salt concentrations and of the relative strengths of binding by SPR and ITC microcalorimetry

Analyte[a]	Surface plasmon resonance								Microcalorimetry K_D
	Salt	Flow rate	k_a	k_d	K_D	K_D eq.[b]	K_D relative to 150 mM salt	Relative to R3[c]	
	mM	$\mu l\ min^{-1}$	$M^{-1}\ s^{-1}$	s^{-1}	M	M			M
Heptasaccharide P_3	150				ND[d]	ND			
	300	10	1.5e3	5.1e-2	3.3e-5	4.8e-5		0.01	
Heptasaccharide P_4	150				ND	ND			
	300	10	3.0e3	5.8e-2	2.0e-5	5.8e-5		0.02	
Octasaccharide 1 P_3	150	10	2.2e3	5.9e-2	2.7e-5	ND		0.01	
	300	40	7.7e3	2.2e-2	2.8e-6	1.9e-5	10	0.13	
Octasaccharide P_4	150				ND	ND			
	300	40	9.8e4	9.4e-2	9.5e-7	1.2e-6		0.38	
Nonasaccharide P_3	150				ND	ND			
	300	10	1.1e3	3.9e-2	3.6e-5	ND		0.01	
E. coli R1 (OS 1)	150	5	1.1e4	4.3e-2	3.9e-6	4.5e-6		0.05	
	300	40	1.6e5	2.8e-2	1.7e-7	7.0e-7	25	2.12	
E. coli R2	150	5	1.7e4	4.3e-3	2.6e-7	ND		0.81	5.5e-9
	300	40	1.4e5	4.6e-3	3.2e-8	ND	8	11.25	
E. coli $R3_{GlcN}$ (OS 2)	150	5	2.7e2	6.6e-3	2.5e-5	ND		0.01	
	300	40	6.4e2	8.6e-3	1.4e-5	ND	1.9	0.03	
E. coli R3 (OS 1)	150	5	1.6e5	3.4e-2	2.1e-7	2.4e-7		1.00	5.6e-8
	300	40	8.7e4	3.1e-2	3.6e-7	3.8e-7	0.6	1.00	
E. coli R4	150	5	1.5e4	4.7e-2	3.2e-6	3.1e-6		0.06	4.8e-8
	300	40	1.6e5	4.8e-2	2.9e-7	3.3e-7	11	1.24	7.4e-8

[a] For structures of analytes see Figs. 1 and 2; *E. coli* $R3_{GlcN}$ refers to the *E. coli* R3 core OS 2 containing Glc*p*N on the side chain heptose.
[b] Determined at steady state equilibrium in SPR.
[c] SPR data compared at the same salt concentration and in relation to the values obtained for oligosaccharide 1 (Fig. 2) from *E. coli* R3.
[d] ND, not determined.

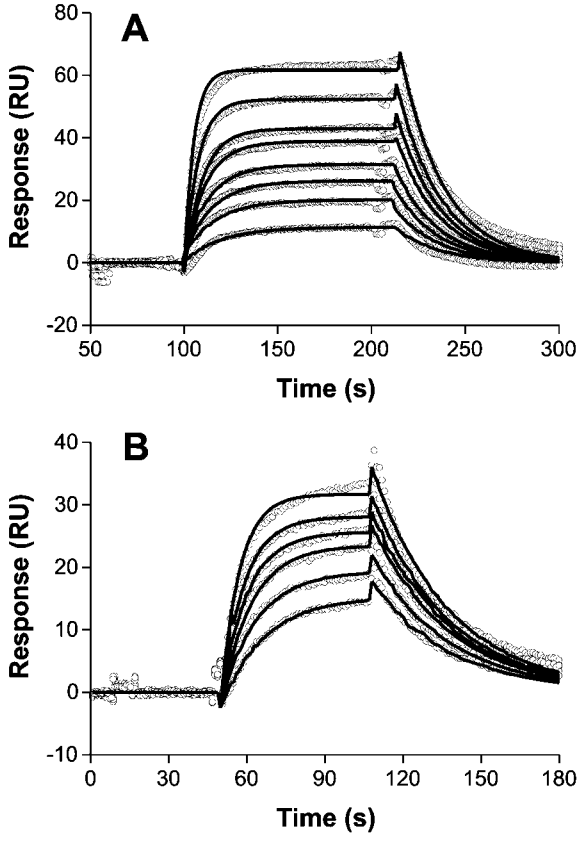

FIG. 8. **Binding of the *E. coli* F2513 (R4 core) oligosaccharide to WN1 222-5 in Hepes-buffered saline containing 150 mM NaCl (*A*) and Hepes-buffered saline containing 300 mM NaCl (*B*).** The oligosaccharide concentration ranges were 0.5–10 μM (*A*) and 0.1–0.3 μM (*B*). Data were globally fitted to a 1:1 interaction model, with the *open circles* representing the data points and the *solid lines* showing the global fit.

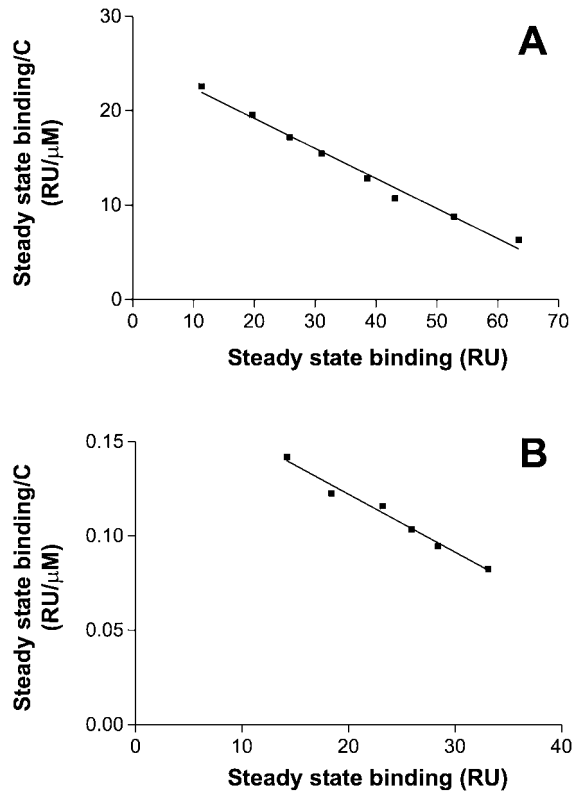

FIG. 9. **Scatchard analysis of equilibrium data for the binding of the *E. coli* F2513 (R4 core) oligosaccharide to WN1 222-5 in Hepes-buffered saline containing 150 mM NaCl (*A*) and Hepes-buffered saline containing 300 mM NaCl (*B*).** The data sets were the same as in Fig. 8.

fere with the binding of highly negatively charged oligosaccharides with WN1 222-5. At 150 mM NaCl, measurements were only possible at a low flow rate of 5 μl min^{-1}, increasing risk of mass transport limitations. Nevertheless, at this flow rate, high quality data sets were obtained, and the almost perfect fit excluded the possibility that mass transport compromised the data. Measurements at 300 mM NaCl allowed data collection at higher flow rates and gave lower K_D values relative to the 150

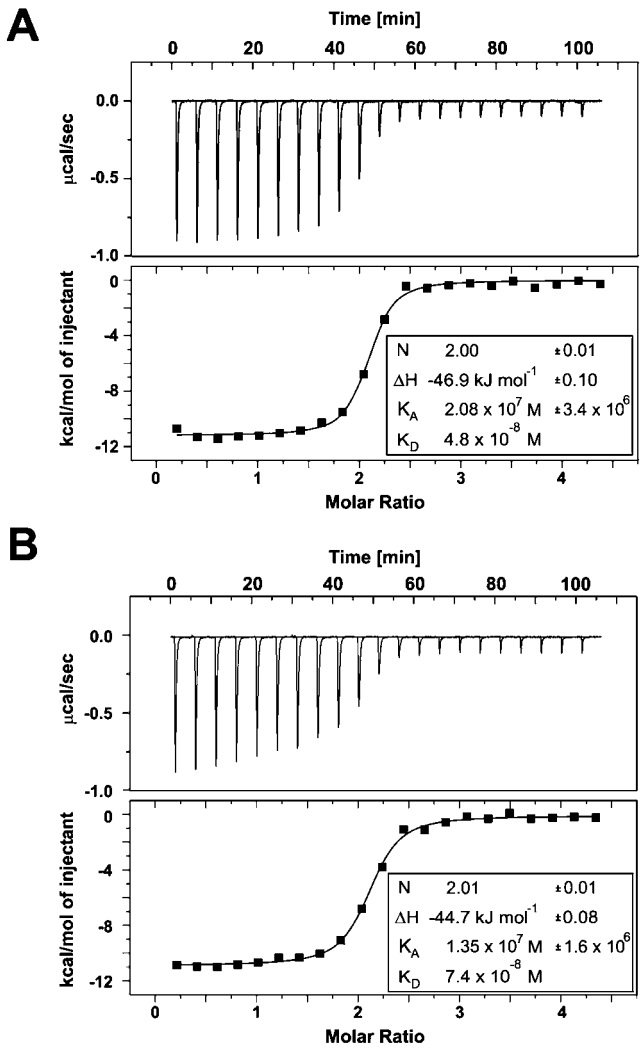

FIG. 10. **ITC microcalorimetry of WN1 222-5 binding to the *E. coli* F2513 (R4 core) oligosaccharide at 150 mM (*A*) and 300 mM (*B*) salt concentration.** *N* represents the number of binding sites; K_A and K_D are affinity and dissociation constants, respectively; and ΔH is the enthalpy of binding.

mM NaCl data. However, the K_D values were still ~10-fold higher than those obtained by microcalorimetry. These results suggest that the negatively charged matrix on Biacore sensor chips may present a problem with highly negatively charged analytes such as LPS core oligosaccharides. However, a comparison of the SPR data, the microcalorimetry data, and the inhibition ELISA data indicated that ranking, by SPR, of analytes with respect to binding affinities is valid.

It has been shown that WN1 222-5 can protect mice from death in an experimental model of septic shock and that this mAb reduces the levels of proinflammatory cytokines such as tumor necrosis factor-α and interleukin-1, which are the key mediators of septic shock, upon LPS stimulation in whole blood (26). The underlying mechanism of the protective effect is probably the removal of endotoxin from the circulation, preventing LPS-responsive cells from stimulation. However, since macrophages are one of the prime sources of proinflammatory cytokines, it is not known why LPS bound to WN1 222-5 is unable to stimulate their release, in particular since the biologically active component is the lipid A portion of LPS, and fatty acids are not part of the epitope, which would explain the protective effect.

Many of the studies aimed at the induction of cross-reactive antibodies have used *E. coli* J-5 or bacteria producing Re-type LPS (*E. coli* F515, *S. enterica* sv. Minnesota R595), which contain only an α2→4-Kdo disaccharide attached to the lipid A. All of these attempts failed, which is now understood. In smooth-type LPS, which is the main form in wild-type *E. coli* bacteria, the presence of the O-antigen and further sugars of the inner and outer core probably mask the Kdo and lipid A part of the molecule, which are therefore inaccessible for antibodies. In LPS from *E. coli* J-5, octasaccharide P_4, the molecule with the highest affinity, accounts for only 25% of the LPS (14). All other molecules possess either severely reduced or very low affinities. Therefore, immunization with *E. coli* J-5 bacteria or LPS may not lead to the induction of cross-reactive antibodies that recognize the epitope commonly present in *E. coli*, *Salmonella*, and *Shigella* LPS. Also, it may be speculated that antibodies raised by immunization with *E. coli* J-5 LPS are either specific for this type of LPS or are inhibited by the presence of outer core sugars.

WN1 222-5 was isolated by immunization of mice with a combination of LPS of all different *E. coli* core types and then selected for cross-reactivity (9). The antibody is therefore able to recognize its epitope in the presence of an outer core, which has a positive effect on the interaction and results in a higher affinity. A strategy aimed at the induction of cross-reactive antibodies by vaccination with conjugated LPS of the *E. coli* F576 (R2 core) is recommended, since WN1 222-5 showed the highest affinity for this structure in all assays employed in this study.

The conjugation of bacterial polysaccharides and LPS is of special interest, because such neoglycoconjugates are promising candidates for safe and immunogenic conjugate vaccines (27–29). The conjugation to proteins transforms T-cell-independent polysaccharide antigens into T-cell-dependent antigens leading to B-cell memory and thus improvement of the immune response on repeated immunizations. Such conjugate vaccines are currently in use or being tested in clinical trials for *Hemophilus influenzae* type b, *Neisseria meningitidis*, and *Streptococcus pneumoniae* (28). The successful use of the *H. influenzae* type b vaccine has prompted several studies that are currently under way for several other bacterial pathogens such as *Shigella* and *Vibrio cholerae* O139 (29). The finding

TABLE III
Determination of inhibitory concentrations of deacylated enterobacterial LPS oligosaccharides with WN1 222–5 by ELISA inhibition

Inhibitor[a]	Amount of inhibitor yielding 50% ELISA inhibition			
	E. coli R3-BSA solid phase antigens[b]		*E. coli* R3 OS1-BSA solid phase antigens[b]	
	ng/well	*µM*	*ng/well*	*µM*
E. coli R3 (1% HAc)	5000	ND[c]	ND	ND
E. coli J-5 (1% HAc)	5000	ND	ND	ND
E. coli R3 (KOH)	20	ND	ND	ND
E. coli R3 (OS 1)	10	0.1	19	0.2
E. coli $R3_{GlcN}$ (OS 2)	1250	9.7	5000	39.0
Heptasaccharide P_3	ND	ND	2500	31.9
Heptasaccharide P_4	156	1.9	1250	15.0
Octasaccharide 1 P_3	ND	ND	312	3.6
Octasaccharide P_4	5	0.1	10	0.1
Nonasaccharide P_3	1250	12.7	5000	51.0

[a] For structures of inhibitors see Figs. 1 and 2; *E. coli* R3 OS 1 and OS 2 refer to the core oligosaccharides without and with Glc*p*N on the side chain heptose, respectively. *E. coli* R3 (1% HAc) and (KOH) are mixtures of oligosaccharides obtained after mild acid and alkaline degradation, respectively.

[b] Solid phase antigens were prepared from *E. coli* R3 LPS after deacylation under alkaline conditions and conjugation to BSA. *E. coli* R3-BSA contains the mixture of LPS oligosaccharides, whereas *E. coli* R3 OS1-BSA refers to the conjugated purified oligosaccharide.

[c] ND, not determined.

that deacylated LPS of the different *E. coli* and *S. enterica* when conjugated to protein react equally well with mAb WN1 222-5 like whole LPS and bacteria opens the way for vaccine development. The isolation and characterization of oligosaccharides bound by WN1 222-5 and the identification of the epitope recognized provides the basis for further experiments aimed at the characterization of this interaction by NMR such as saturation transfer difference measurements (30) or by x-ray crystallography. Such experiments would lead to a deeper understanding of this interaction and, combined with conformational analysis of endotoxic molecules, eventually to the rational design of a vaccine based on a single oligosaccharide structure protective against infections and septic shock from different Gram-negative pathogens such as *E. coli*, *Salmonella*, *Shigella*, and *Citrobacter*.

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