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

Received for publication, March 21, 2003, and in revised form, April 23, 2003
Published, JBC Papers in Press, April 25, 2003, DOI 10.1074/jbc.M302904200

Sven Müller-Loennies†‡, Lore Brade†‡, C. Roger MacKenzie¶, Franco E. Di Padova**, and Helmut Brade‡

From the †Research Center Borstel, Center for Medicine and Biosciences, Parkallee 22, D-23845 Borstel, Germany, the ‡Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada, and **Novartis Pharma AG, CH-4002 Basel, Switzerland

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 mononclonal antibody (mAb WN1 222-5) regarded as a potential means of treatment. Using whole LPS and a panel of glycoconjugates 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 R3-α-D-Glc-(1→3)-(L-α-D-Hepp- (1→7)-L-α-D-Hepp) 4P-(1→3)-R4 (where R3 represents additional sugars of the outer core and R4 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 GlcPN 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 (KD values) of the monovalent interaction of E. coli core oligosaccharides with WN1 222-5 by surface plasmon resonance and isothermal titration microcalorimetry. Affinity constants (KD values) determined by SPR were in the range of 3.6 × 10⁻⁸ 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 KD 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; 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 sepsis, 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).

Antiserum 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...
A Cross-reactive Epitope of Enterobacterial LPS

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).

For J-5 LPS, five different oligosaccharides, which differ in their carbohydrate structures and phosphate substitution (11), were obtained by deacylation under strong alkaline conditions (4M KOH, 120 °C, 4 h). After 3-fold extraction with chloroform, the mixture of deacylated oligosaccharides was separated by high performance anion exchange chromatography (HPAEC) and the depicted oligosaccharides were obtained from the J-5 mutant. Octasaccharide 1 P₃, 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.

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/petroleum ether extraction (18) and de-acylated by mild hydrazinolysis, followed by de-N-acylation under strong alkaline conditions (4 M KOH, 120 °C, 4 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 NH₄HCO₃ 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 NaNO₂ 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, 0.8 mg; OS 3, 2.3 mg; OS 4, 0.8 mg).

Non-glycosylconjugates—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; glutaraldehyde (25%, electron microscopy grade; Merck) was added (1% final concentration); and the sample was stirred for 4 h at 25 °C under N₂ atmo-
A Cross-reactive Epitope of Enterobacterial LPS

Asialo-glycoprotein 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 µl of NaBH₄ 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% (2.225 ppm) (1H) and dioxane (67.4 ppm) (13C) served as references. 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 CMS 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 5 mM EDTA, 0.005% P-20, and 150 mM or 300 mM NaCl. Surface regeneration was not necessary. Data analysis were performed 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 mixture was incubated overnight at 25°C. Finally, 250 µl of NaBH₄ was added, and the mixture was incubated for 1 h at 4°C in the dark followed by dialysis against water once and three times against PBS, pH 7.2.

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. 6A). 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. 6B, 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 GlcN 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
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\_pN, 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\_pN side chain substitution) was unable to inhibit the interaction between WN1 222-5 and the R3 core oligosaccharide 1 (without Glc\_pN 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 mAb WN1 222-5, we have conjugated each of the oligosaccharides obtained from E. coli J-5 LPS and the newly prepared octasaccharide 1\_P3 to BSA using glutardialdehyde coupling (20). The octasaccharide 1\_P3 conjugate was included to elucidate the importance of phosphate substitution and side chain heptose substitution with Glc\_pN 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 1\_P3 and heptasaccharide 2\_P3, whereas intermediate binding to heptasaccharide 3\_P3 and octasaccharide 1\_P3 was observed. The highest affinity was observed for the interaction of WN1 222-5 and octasaccharide 1\_P3.

In ELISA inhibition (see Table III), nonasaccharide 1\_P3 and heptasaccharide 2\_P3 were both unable to inhibit the interaction between WN1 222-5 and the R3 core-oligosaccharide 1 (without Glc\_pN in the core), whereas heptasaccharide 3\_P3 and octasaccharide 1\_P3 showed inhibitory activity, yielding 50% inhibition values at concentrations of 15 and 3.6 \( \mu \)g/ml, respectively. Octasaccharide 1\_P3 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-
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-GlcP-(1→3)-l-α-d-Hep-(1→7)-l-α-d-Hep P4 (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 P3 of E. coli J-5, or the phosphate at the second heptose, as in octasaccharide 1 P3, 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 P3, or in which the side chain heptose is masked by GlcpN substitution, such as nonasaccharide P3, 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 Glcp-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 P4 using α-glucosidases.
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 $3.2 \times 10^{-9}$ 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$_3$ ($K_D$ = 9.5 x $10^{-7}$ 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 3'-6-Galp 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

![Figure 4](image-url)

**FIG. 4.** $^1$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$_3$.

![Figure 5](image-url)

**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-D-Hep (residue E; for structure see inset in Fig. 4).
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 P3 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-

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 P4, octasaccharide P4, heptasaccharide P, octasaccharide 1 P, and nonasaccharide P, 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 ( ).
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

### Table II

| Analyte | Surface plasmon resonance | Microcalorimetry |
|---------|----------------------------|------------------|
|         | $K_a$ | $K_d$ | $K_D$ | $K_D$ | $K_D$ relative to 150 mM salt | Relative to R3 |
| Heptasaccharide P₃ | 150 | 300 | 10 | 1.5e3 | 5.1e-2 | 3.3e-5 | 4.8e-5 | 0.01 |
| Heptasaccharide P₄ | 150 | 300 | 10 | 3.0e3 | 5.8e-2 | 2.0e-5 | 5.8e-5 | 0.02 |
| Octasaccharide 1 P₃ | 150 | 300 | 40 | 7.7e3 | 2.2e-2 | 2.8e-6 | 1.9e-5 | 0.13 |
| Octasaccharide P₇ | 150 | 300 | 40 | 9.8e4 | 9.4e-2 | 9.5e-7 | 1.2e-6 | 0.38 |
| Nonasaccharide P₇ | 150 | 300 | 10 | 1.1e3 | 3.9e-2 | 3.8e-5 | ND | 0.01 |
| *E. coli* R1 (OS 1) | 150 | 300 | 5 | 1.1e4 | 4.3e-2 | 3.9e-6 | 4.5e-6 | 0.05 |
| *E. coli* R2 | 150 | 300 | 40 | 1.6e5 | 2.8e-2 | 1.7e-7 | 7.0e-7 | 25 | 2.12 |
| *E. coli* R3 (OS 2) | 150 | 300 | 5 | 1.7e4 | 4.3e-3 | 2.6e-7 | ND | 0.81 | 5.5e-9 |
| *E. coli* R4 | 150 | 300 | 5 | 1.6e4 | 4.7e-2 | 3.2e-6 | 2.4e-6 | 1.00 | 5.6e-8 |
| *E. coli* R3GlcN (OS 1) | 150 | 300 | 10 | 1.1e3 | 4.3e-2 | 3.8e-5 | ND | 0.01 |
| *E. coli* R3GlcN (OS 2) | 150 | 300 | 5 | 1.6e5 | 3.4e-2 | 2.1e-7 | 2.4e-7 | 1.00 | 5.6e-8 |
| *E. coli* R4 | 150 | 300 | 5 | 1.6e4 | 4.7e-2 | 3.2e-6 | 2.4e-6 | 0.06 | 4.8e-8 |
| Nonasaccharide P₇ | 150 | 300 | 10 | 1.1e3 | 3.9e-2 | 3.8e-5 | ND | 0.01 |
| *E. coli* R1 (OS 1) | 150 | 300 | 5 | 1.1e4 | 4.3e-2 | 3.9e-6 | 4.5e-6 | 0.05 |
| *E. coli* R2 | 150 | 300 | 40 | 1.6e5 | 2.8e-2 | 1.7e-7 | 7.0e-7 | 25 | 2.12 |
| *E. coli* R3 (OS 2) | 150 | 300 | 5 | 1.7e4 | 4.3e-3 | 2.6e-7 | ND | 0.81 | 5.5e-9 |
| *E. coli* R4 | 150 | 300 | 5 | 1.6e4 | 4.7e-2 | 3.2e-6 | 2.4e-6 | 1.00 | 5.6e-8 |
| *E. coli* R3GlcN (OS 1) | 150 | 300 | 10 | 1.1e3 | 4.3e-2 | 3.8e-5 | ND | 0.01 |
| *E. coli* R3GlcN (OS 2) | 150 | 300 | 5 | 1.6e5 | 3.4e-2 | 2.1e-7 | 2.4e-7 | 1.00 | 5.6e-8 |

a For structures of analytes see Figs. 1 and 2; *E. coli* R3GlcN refers to the *E. coli* R3 core OS 2 containing GlcP 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.
A Cross-reactive Epitope of Enterobacterial LPS

**TABLE III**

| Inhibitor<sup>a</sup> | Amount of inhibitor yielding 50% ELISA inhibition<sup>b</sup> |
|----------------------|-------------------------------------------------|
|                      | E. coli R3 BSA solid phase antigen<sup>c</sup> | E. coli R3 OS1-BSA solid phase antigen<sup>c</sup> |
|                      | ng/well | μM | ng/well | μM |
| E. coli R3 (1% HAc)  | 5000    | ND | 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 (OS 2)   | 1250    | 9.7| 5000    | 39.0|
| Heptasaccharide P<sub>3</sub> | ND | ND | 2500 | 31.9|
| Heptasaccharide P<sub>4</sub> | 156 | 1.9| 1250 | 15.0|
| Octasaccharide P<sub>4</sub> | ND | 312 | 3.6|
| Octasaccharide P<sub>6</sub> | 5      | 0.1| 10     | 0.1|
| Nonasaccharide P<sub>8</sub> | 1250 | 12.7| 5000 | 51.0|

<sup>a</sup> For structures of inhibitors see Figs. 1 and 2; E. coli R3 OS1 and OS2 refer to the core oligosaccharides without and with GlcN 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.

<sup>b</sup> 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.

<sup>c</sup> ND, not determined.

LPS (E. coli F515, *S. entérica* 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<sub>4</sub>, 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
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.

Acknowledgments—We gratefully acknowledge Drs. Otto Holst and E. Vinogradov for the kind gift of E. coli core oligosaccharides R1 and R2. We thank T. Hirama, V. Susott, S. Cohrs, A. Denzin, G. Lehwark, and G. von Busse for technical assistance.

REFERENCES

1. Vaara, M. (1999) in Endotoxin in Health and Disease (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C., eds) pp. 31–38, Marcel Dekker, Inc., New York
2. Holst, O. (1999) in Endotoxin in Health and Disease (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C., eds) pp. 115–154, Marcel Dekker, Inc., New York
3. Levy, O., and Elsbach, P. (2001) Curr. Infect. Dis. Rep. 3, 407–412
4. Fenton, M. J., and Golenbock, D. T. (1998) J. Leukocyte Biol. 64, 25–32
5. Pollack, M., and Ohl, C. A. (1999) in Pathology of Sepsis and Septic Shock (Rietschel, E. T., and Wagner, H., eds) pp. 275–297, Springer, Heidelberg, Germany
6. Baumgartner, J.-D., Heumann, D., and Glauser, M.-P. (1999) in Endotoxin in Health and Disease (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C., eds) pp. 865–876, Marcel Dekker Inc., New York
7. Müller-Lönnies, S., Di Padova, F. E., Brade, L., Heumann, D., and Rietschel, E. T. (2000) in Fighting Infection in the 21st Century (Andrew, P. W., Oyston, P., Smith, G. L., and Stewart-Tull, D. E., eds) pp. 143–178, Blackwell Science Ltd., Oxford
8. Brande, A. I., and Douglas, H. (1972) J. Immunol. 108, 505–512
9. Di Padova, F. E., Brade, H., Barclay, G. R., Poxton, I. R., Liefh, E., Schuetze, E., Kocher, H. P., Ramsay, G., Schreier, M. H., and McClelland, D. B. (1993) Infect. Immun. 61, 3863–3872
10. Elbein, A. D., and Heath, E. C. (1965) J. Biol. Chem. 240, 1919–1925
11. Holst, O., Müller-Lönnies, S., Lindner, B., and Brade, H. (1993) Eur. J. Biochem. 214, 695–701
12. Brade, L., Holst, O., and Brade, H. (1993) Infect. Immun. 61, 4514–4517
13. Müller-Lönnies, S., Grimmecke, D., Brade, L., Lindner, B., Kosma, P., and Brade, H. (2002) J. Endotoxin Res. 8, 295–305
14. Müller-Lönnies, S., Holst, O., Lindner, B., and Brade, H. (1999) Eur. J. Biochem. 260, 235–249
15. Vinogradov, E. V., Van Der, D. K., Thomas-Oates, J. E., Meshkov, S., Brade, H., and Holst, O. (1999) Eur. J. Biochem. 261, 629–639
16. Müller-Lönnies, S., Lindner, B., and Brade, H. (2002) Eur. J. Biochem. 269, 5982–5991
17. Olothorn, M. M., Petersen, B. O., Schlecht, S., Havenerkamp, J., Bock, K., Thomas-Oates, J. E., and Holst, O. (1998) J. Biol. Chem. 273, 3817–3829
18. Galanos, C., Luderitz, O., and Westphal, O. (1969) Eur. J. Biochem. 9, 245–249
19. Vinogradov, E., Cedzynski, M., Zollikowski, A., and Swierza, A. (2001) Eur. J. Biochem. 268, 1722–1729
20. Brade, L., Brunnenmann, H., Ernst, M., Pu, Y., Holst, O., Kosma, P., Naher, H., Persson, K., and Brade, H. (1994) FEMS Immunol. Med. Microbiol. 8, 27–41
21. Müller-Lönnies, S., Brade, L., and Brade, H. (2002) Eur. J. Biochem. 269, 1237–1242
22. Holst, O., and Brade, H. (1991) Carbohydr. Res. 219, 247–251
23. Czyler, M., Rose, D. R., and Bunde, D. R. (1991) Science 253, 442–445
24. Jeffrey, P. D., Rajorath, J., Chang, C. Y., Yelton, D., Hilmstrom, I., Hilmstrom, K. E., and Sheriff, S. (1995) Nat. Struct. Biol. 2, 466–471
25. Kastowsky, M., Gutberlet, T., and Bradaczeski, H. (1992) J. Bacteriol. 174, 4788–4806
26. Bailat, S., Heumann, D., Le Roy, D., Baumgartner, J. D., Rietschel, E. T., Glauser, M. P., and Di Padova, F. (1997) Infect. Immun. 65, 811–814
27. Makela, P. H. (2000) FEMS Microbiol. Rev. 24, 9–20
28. Tan, T. Q. (2000) Curr. Opin. Microbiol. 3, 502–507
29. Bouteinier, A., Villeneuve, S., Nato, F., Dassy, B., and Fourrier, J. M. (2001) Infect. Immun. 69, 3488–3493
30. Meyer, B., Weimar, T., and Peters, T. (1997) Eur. J. Biochem. 246, 705–709
Identification of a Cross-reactive Epitope Widely Present in Lipopolysaccharide from Enterobacteria and Recognized by the Cross-protective Monoclonal Antibody WN1 222-5

Sven Müller-Loennies, Lore Brade, C. Roger MacKenzie, Franco E. Di Padova and Helmut Brade

J. Biol. Chem. 2003, 278:25618-25627.
doi: 10.1074/jbc.M302904200 originally published online April 25, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302904200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 25 references, 9 of which can be accessed free at http://www.jbc.org/content/278/28/25618.full.html#ref-list-1