Antigenic Potential of a Highly Conserved Neisseria meningitidis Lipopolysaccharide Inner Core Structure Defined by Chemical Synthesis

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SUMMARY

Neisseria meningitidis is a leading cause of bacterial meningitis worldwide. We studied the potential of synthetic lipopolysaccharide (LPS) inner core structures as broadly protective antigens against N. meningitidis. Based on the specific reactivity of human serum antibodies to synthetic LPS cores, we selected a highly conserved LPS core tetrasaccharide as a promising antigen. This LPS inner core tetrasaccharide induced a robust IgG response in mice when formulated as an immunogenic glycoconjugate. Binding of raised mouse serum to a broad collection of N. meningitidis strains demonstrated the accessibility of the LPS core on viable bacteria. The distal trisaccharide was identified as the crucial epitope, whereas the proximal Kdo moiety was immunodominant and induced mainly nonprotective antibodies that are responsible for lack of functional protection in polyclonal serum. Our results identified key antigenic determinants of LPS core glycan and, hence, may aid the design of a broadly protective immunization against N. meningitidis.

INTRODUCTION

N. meningitidis is an encapsulated human bacterial pathogen that causes meningitis and sepsis worldwide. Vaccines based on capsular polysaccharides are available against invasive meningococcal serogroups A, C, W, and Y, but not for B, as its capsule consists of autoantigenic α-(2→8)-polysialic acid (Finne et al., 1983; Granoff, 2010). Thus, vaccines based on noncapsular antigens are needed to limit N. meningitidis B infections, with globally 20,000 to 80,000 cases per year, especially in developed countries (Hedari et al., 2014). Recently, a multicomponent vaccine 4CMenB, based on recombinant proteins and outer membrane vesicles, was developed by Novartis (Bexsero) that is protective against 88% of N. meningitidis B strains (Frosi et al., 2013). The composition of 4CMenB was adjusted to target serogroup B strains. The vaccine components of 4CMenB are present in N. meningitidis isolates of other serogroups and bear the potential to protect against non-B serogroups (Hong et al., 2013). Alternatively, lipopolysaccharide (LPS) presents a promising subcapsular antigen that might elicit broader reactivity against all N. meningitidis strains. The LPS is also called lipooligosaccharide because of the absence of O-polysaccharide and is anchored via lipid A in the outer membrane (Nikaido, 2003; Raetz and Whitfield, 2002). The meningococcal LPSs only show a core oligosaccharide that is subdivided into inner and outer cores. The inner core consists of two Kdo (3-deoxy-α-D-manno-oct-2-ulosonic acid) and two Hep (L-glycero-D-manno-heptose) residues (Figure 1). The LPS outer core contains mostly of hexoses building up variable α chain extensions from HepI (Figure 1, inset). In addition, β chain and γ chain extensions and a phosphoethanolamine (PEtN) modification on HepII are possible. These diverse outer core compositions determine 12 distinct immunotypes of N. meningitidis (L1–L12) (Choudhury et al., 2008; Nikaido, 2003; Plasted et al., 1999). The immunotype expression is subjected to phase variation (Berrington et al., 2002; Zhu et al., 2002). L1–L8 immunotypes are associated with serogroups B and C, and L9 immunotype is common in serogroups A, B, and C, whereas L10–L12 immunotypes are found in serogroup A (Mistretta et al., 2010). In contrast, the LPS inner core is highly conserved because inner core glycosyltransferase genes are not subjected to phase variation (Kahler et al., 2005). The potential of the LPS inner core as subcapsular antigen was evaluated earlier using purified LPS from a ΔgalE mutant of the widely studied N. meningitidis B strain MC58 (immunotype L3; Figure 1, inset). The ΔgalE LPS shows an inner core with an α chain extension of β-(1→4)-linked Glc on HepI, with α-(1→2)-linked GlcNAc as a γ chain extension and PEtN modification at the 3-position of HepII. A monoclonal antibody (mAb) B5 raised against the purified ΔgalE LPS bound to 76% of N. meningitidis B strains and to 70% of a collection of N. meningitidis strains of serogroups A, C, W, X, Y, and Z. All these strains share the common ΔgalE LPS core epitope with PEtN at the 3-position of HepII (Plasted et al., 1999). mAb B5 has been shown to promote serum
bactericidal activity (SBA) in a gold standard surrogate assay for in vivo protection against *N. meningitidis* infections (Plested et al., 2001, 2003). Human antibodies specific for LPS inner cores showed bactericidal and opsonic activity as well (Ja¨kel et al., 2008; Plested et al., 2001). However, conjugation of detoxified LPS to a carrier protein that is required to induce a robust immune response is not straightforward. Aggregation and low solubility due to the amphiphilic character of purified LPS

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**Figure 1. Library of 12 Synthetic LPS Core Structures**

Natural LPS structure immunotype L3 of *N. meningitidis* (inset) and synthetic LPS core structures from *N. meningitidis* (1), *Yersinia pestis* (8, 11), *Haemophilus influenzae* (9), *Proteus* (10), *Chlamydia* (12), and their truncated derivatives (2–7).
impede reproducible glycoconjugate synthesis. Decaylation and incorporation of functional groups for covalent linkage to carrier proteins are performed on purified LPS prior to conjugation but generate neoepitopes that may elicit nonspecific antibodies (Cox et al., 2010a, 2010b; St Michael et al., 2014). Furthermore, purified LPS offers a limited possibility for antigen refinement due to its restriction to sequentially expressed LPS core structures on engineered knockout mutants. In contrast, chemical synthesis offers virtually unlimited structural variability that allows precise definition and manipulation of the antigen.

We used a combination of chemical synthesis and glycan array screening to define a highly conserved portion of the LPS inner core structure as a potential antigen candidate. For that purpose, a library of species-specific LPS inner core oligosaccharides was synthesized (Figure 1). Based on glycan array screening of these synthetic oligosaccharides with human sera, the conserved LPS inner core structure of all N. meningitidis immunotypes α-D-GlcNac-(1→2)-L-α-D-Hep-(1→3)-L-α-D-Hep-(1→5)-α-Kdo (Figure 1) was chosen for further immunological evaluation. Therefore, this tetrascarhide was conjugated to the carrier protein CRM, a nontoxic diphtheria toxin variant commonly used for glycoconjugate vaccines. The resulting glycoconjugate generated an antibody response against the tetrascarhide and a collection of closely related synthetic LPS core analogs in mice. Binding of serum antibodies to a broad collection of viable wild-type (wt) N. meningitidis strains expressing diverse immunotypes demonstrated the accessibility of the LPS inner core on the bacterial cell surface. The structural requirements of the synthetic antigen to elicit protective antibodies were assessed with a combination of glycan array, surface plasmon resonance (SPR), and immunoassays. We report the epitope mapping of bacterial cell surface. The structural requirements of the synthetic antigen to elicit protective antibodies were assessed with a combination of glycan array, surface plasmon resonance (SPR), and immunoassays. We report the epitope mapping of bacterial cell surface. The structural requirements of the synthetic antigen to elicit protective antibodies were assessed with a combination of glycan array, surface plasmon resonance (SPR), and immunoassays. We report the epitope mapping of bacterial cell surface. The structural requirements of the synthetic antigen to elicit protective antibodies were assessed with a combination of glycan array, surface plasmon resonance (SPR), and immunoassays. We report the epitope mapping of bacterial cell surface. The structural requirements of the synthetic antigen to elicit protective antibodies were assessed with a combination of glycan array, surface plasmon resonance (SPR), and immunoassays. We report the epitope mapping of bacterial cell surface. The structural requirements of the synthetic antigen to elicit protective antibodies were assessed with a combination of glycan array, surface plasmon resonance (SPR), and immunoassays.

RESULTS AND DISCUSSION

Synthesis of LPS Inner Core Oligosaccharides 5 and 6

The LPS inner core oligosaccharides 1–4, 7–10 (Yang et al., 2012, 2013), 11 (Anish et al., 2013), and 12 (Guo, 2011) were prepared as previously described by relying on de novo and diversity-oriented synthetic strategies (Figure 1). However, synthesis of disaccharide 6 and trisaccharide 5 can be conducted with less sophisticated building blocks. L-α-D-Hep-(1→3)-L-α-D-Hep disaccharide 6 and α-D-GlcNac-(1→2)-L-α-D-Hep-(1→3)-L-α-D-Hep trisaccharide 5 were assembled from building blocks 13–15 (Figure 2). Hep building blocks 14 and 15 were prepared by using our previously developed synthetic procedures (Yang et al., 2012, 2013). Hep building block 13 was accessed by introducing an N-benzyl-N-benzylxoycarbonylpentyl linker (Mong et al., 2003; Noti et al., 2006) on the reducing end of Hep intermediate 16 that was, in turn, obtained by de novo synthesis (Ohara et al., 2010). Hep intermediate 16 was converted into the corresponding N-phenyl trifluoroacetimidate (Yu and Tao, 2001, 2002) 17 upon selective anomic delevulnoloylation and N-phenyl trifluoroacetimidate formation (78% yield over two steps). Glycosylation of linker 18 with N-phenyl trifluoroacetimidate 17 under the catalysis of TMSOTf in a mixture of dichloromethane and ether produced the desired α-linked monosaccharide 19 in 72% yield. In the presence of hydrazine and acetic acid, the O-Lev group in 19 was selectively removed to afford Hep building block 20 in 87% yield. The coupling of Hep 14 and 13 promoted by TMSOTf gave the desired α-(1→3)-linked disaccharide that was treated with HF-pyridine to afford disaccharide 21 in 65% yield over two steps. Saponification of the acetyl groups in 20 with sodium methoxide followed by hydrogenolysis over palladium on carbon (Pd/C) provided L-α-D-Hep-(1→3)-L-α-D-Hep disaccharide 6 in 75% yield over two steps. Coupling of disaccharide 15 and Hep 13 promoted by TMSOTf in a mixture of dichloromethane and ether gave the desired α-(1→3)-linked trisaccharide. Treatment of the newly formed trisaccharide with thiacetic acid in pyridine allowed separation of trisaccharide 21 from the remaining derivative of monosaccharide 13 at this step, thus affording acetamide 21 in 72% yield over two steps. Global deprotection of 21 involved TBPS cleavage, saponification, and hydrogenolysis over Pd/C to provide α-D-GlcNAc-(1→2)-L-α-D-Hep-(1→3)-L-α-D-Hep trisaccharide 5 (33% yield over three steps).

Naturally Occurring Human Antibodies Bind Selectively to Synthetic LPS Inner Core Oligosaccharides

N. meningitidis colonizes the nasopharyngeal mucosa asymptomatically, a state referred to as carriage occurring in 35% of healthy young adults, and opportunistically invades and infects exclusively humans (Caugant et al., 2007). Mapping binding preferences of natural antibodies raised by asymptomatic carriers and infected patients provides direct evidence for their immunogenicity in vivo that helps to define the antigenic epitopes. Naturally occurring immunoglobulin (Ig)G and IgM antibodies specific for ΔgalE LPS core glycolipid were earlier detected in healthy and infected persons via ELISA, demonstrating the immunogenicity of the ΔgalE LPS core (Plestis et al., 2000). To examine whether antibodies specific for synthetic LPS core structures are present in humans who have been exposed to N. meningitidis, we performed glycan array screenings of sera from convalescent patients who recovered from N. meningitidis infection (n = 11), asymptomatic N. meningitidis carriers (n = 11), and healthy individuals (n = 15) (Hubert et al., 2013; Figure 3). Glycan array analysis revealed that IgG antibodies in human sera showed exclusive binding to meningooccal tetrascarhide 1 and trisaccharide 5, while there was only weak binding to trisaccharide 2. Trisaccharide 2 represents a highly conserved LPS inner core of many Gram-negative bacteria. Low binding to 2, compared to 1 and 5, indicates the importance of the distal trisaccharide 5 for antibody recognition of the N. meningitidis LPS core. Weak binding to the LPS cores of Y. pestis 8, H. influenzae 9, and bacteria of genus Proteus 10 revealed the specificity of natural antibodies for N. meningitidis LPS core glycans. The same trend was observed for IgM antibodies (data not shown). No significant differences between healthy individuals and convalescent patients or asymptomatic N. meningitidis carriers were detected. Discrimination between these groups was based on mucosal swabs revealing the current N. meningitidis colonization status of each individual. Possible previous colonization was not considered. Human sera screening revealed the
presence of antibodies specific for synthetic LPS cores 1 and 5 that appear to be highly antigenic.

**Immunological Evaluation of Synthetic LPS Core Tetrasaccharide 1 from N. meningitidis**

Based on the results of the human serum antibody screening, the immunogenicity of synthetic LPS core tetrasaccharide 1 was tested in a mouse model. Tetrasaccharide 1 was selected over trisaccharide 5 to elicit antibodies with broader specificities (Evenberg et al., 1992; Pozsgay et al., 1999). In addition, the immunogenic potential of charged motifs in oligosaccharide antigens justified the selection of a synthetic target containing a Kdo moiety (Adamo et al., 2012; Palusiak et al., 2014). Most carbohydrates are poorly immunogenic and fail to induce long-lasting memory response. In order to recruit T cell help, we conjugated 1 to carrier protein CRM197 using the previously described di-N-succinimidyl adipate (DSAP) spacer (Brocké et al., 2009, 2011), generating tetrasaccharide 1-CRM197 conjugate 22 (Figures S1A–S1C available online) (Anish et al., 2013; Martin et al., 2013). To investigate the antigenic potential of tetrasaccharide 1, three groups of mice (n = 6) were immunized with different doses of conjugate 22 corresponding to 3 μg, 2 μg, and 1 μg of tetrasaccharide 1 in the presence of a human-approved adjuvant, Alum Alhydrogel (Alum) (Clements and Griffiths, 2002; Figure 4). Mice (n = 6) immunized with an amount of 22 containing 3 μg antigen 1 formulated in Freund’s adjuvant (FA) were used as the positive control because of the promotion of strong carbohydrate-specific immune responses by this adjuvant (Anish et al., 2013; Fu et al., 1992; Martin et al., 2013). The control group (n = 6) was sham-immunized with an equal volume of sterile PBS and showed no anti-tetrasaccharide 1 response. Each mouse received priming and boosting of 22 formulated with the respective adjuvant. A dose-dependent anti-tetrasaccharide 1 response was observed in all mice immunized with Alum-formulated glycoconjugate 22 at day 15 and day 36 (Figure 4). Antibodies against the carrier protein 28 (Figure S1G) and spacer moiety 27 were also observed (Figure 4). Increased fluorescence intensities were detected for BSA-[spacer-GlcNAc] 16 compared to BSA-[spacer-DiMan] 21 due to GlcNAc binding antibodies (Figure S1E). A dose of conjugate 22 containing 1 μg tetrasaccharide 1 was sufficient to elicit a carbohydrate-specific immune response. We observed increased antibody levels after boosting for all groups immunized with 22, demonstrating successful recruitment of T cell help (day 36). Furthermore, we were interested in the generation of a long-lasting memory response. Therefore, mice were left untreated for 224 days until antibody levels subsided before the animals were reimmunized with one fifth of the priming dose at day
Accessibility of Conserved LPS Core Tetrasaccharide 1

On the cell surface of *N. meningitidis*, tetrasaccharide 1 is presented as a subcapsular antigen that is modified by several side chains (Figure 1, inset). To investigate whether antibodies generated against synthetic tetrasaccharide 1 are able to recognize the natural LPS core, we performed immunolabeling of heat-inactivated *N. meningitidis* strains with postimmune sera generated against conjugate 22 administered with Alum elicited robust primary, secondary, and long-lasting memory IgG responses and revealed the immunogenicity of this glycan.

Accessibility of Conserved LPS Core Tetrasaccharide 1 on Viable *N. meningitidis*

On the cell surface of *N. meningitidis*, tetrasaccharide 1 is presented as a subcapsular antigen that is modified by several side chains (Figure 1, inset). To investigate whether antibodies generated against synthetic tetrasaccharide 1 are able to recognize the natural LPS core, we performed immunolabeling of heat-inactivated *N. meningitidis* (27 different strains) with postimmune sera generated against conjugate 22 administered with FA (hereinafter referred to as the “FA group”), as these mice produced the highest IgG levels against 1. Preimmune sera served as the control. Confocal fluorescence microscopy showed weak IgG binding for preimmune sera, while immunolabeling with postimmune sera revealed strong IgG binding to *N. meningitidis* bacteria visualized by bright fluorescein isothiocyanate (FITC) fluorescence (Figure 5A).

Fluorescence signals observed on the bacterial surfaces indicated that antibodies generated against synthetic tetrasaccharide 1 recognized the LPS core on heat-inactivated *N. meningitidis*. Immunolabeling of the encapsulated *N. meningitidis* B wt strain MC58 (used here as a positive control), and its LPS-free ΔpxA mutant was quantified by flow cytometry (Figure S2A). Postimmune sera generated against 22 containing 3 μg of antigen 1 formulated with Alum (hereinafter referred to as the “Alum group”), and postimmune sera of the FA group showed significant binding to MC58 wt compared to MC58 LPS-free ΔpxA mutant. This demonstrates that generated serum antibodies bind to the LPS core of heat-inactivated *N. meningitidis* bacteria. As inactivation may alter the cell-surface structures and expose buried inner core epitopes, we immunolabeled viable, noninactivated *N. meningitidis* strains in an ELISA-based assay. As observed for heat-inactivated strains, postimmune sera of the Alum group showed significantly higher binding to viable MC58 wt compared to LPS-free ΔpxA mutant (Figure 5B) (p = 0.0000028). This observation demonstrates that the LPS inner core on noninactivated strains is accessible to antibodies generated against synthetic tetrasaccharide 1. Binding to representative strains of all 12 immunotypes was observed, revealing the presence and accessibility of conserved tetrasaccharide 1 by postimmune sera of the Alum group (Figure 5B). LPS immunotypes differ in length of α chain as well as modification of HepII with PEtN (3- or 6-position) and/or Glc. In an earlier immunization study with ΔgalE mutant expressing LPS with a PEtN at the 3-position of HepII, the murine mAb B5 was elicited, which recognized only strains expressing this particular PEtN modification (Plested et al., 1999). However, the synthetic tetrasaccharide 1 used in our approach lacks PEtN modification but elicited antibodies that bound to immunotypes expressing PEtN modification at the 3-position (immunotype L1, L3, L7, or L8) (Verheul et al., 1994) or 6-position (immunotype L2, L4, or L6) (St Michael et al., 2009; Weynants et al., 2009) (Figure 5B). Furthermore, the immunotype L5, which is free of PEtN modification, was also bound by postimmune sera of the Alum group. We systematically mapped the epitope recognized by antibodies on the bacterial surface by testing a collection of mutated *N. meningitidis* MC58 strains expressing sequentially truncated LPS structures (Figure 6A) (Jennings et al., 1995; Kurzai et al., 2005; Rahman et al., 2001). Postimmune sera of the Alum group showed significant binding to full-length and to all truncated LPSs. It is interesting that the Δpgm and ΔgalE mutants showed the highest binding compared to other mutants. The LPS core Δpgm mutant closely resembles the synthetic tetrasaccharide 1 (Figure 1). Increased antibody binding to these mutants is in accordance with observations made in earlier immunization studies with detoxified LPS core glycoconjugates that revealed strongest reactivity to homologous strains (Cox et al., 2010a; St Michael et al., 2014). The enhanced recognition of Δpgm and ΔgalE mutants might be the result of optimal exposure and accessibility of the α- GlcNAC-(1→2)-[PEtN-3]-α-Hept-(1→3)-L-α-Hept motif. Furthermore, binding analysis of natural human
antibodies against synthetic LPS cores showed a binding preference to distal trisaccharide 5 (Figure 3). The importance of α-GlcNAc was proven by the significantly decreased binding (p = 0.00019) to the Δrfak mutant that lacks this unit (Figure 6A).

For postimmune sera of the FA group, weak but significant binding to viable MC58 wt, truncated Δlst, and ΔgtA LPS mutants was observed (Figure S2C). Similarly to sera of the Alum group, sera of FA group showed increased binding to Δpgm and ΔαgalE mutants due to expression of closely related LPS core structures to tetrasaccharide 1. However, FA group sera did not recognize further truncated LPS core of Δrfak and Δrfaf mutants. Overall binding to viable N. meningitidis was lower compared to that of the Alum group. Therefore, FA group sera were not further considered in the following studies with viable bacteria.

Postimmune sera of the Alum group showed significant binding to N. meningitidis strains of serogroups A, B, C, W, Y, X, and E and a capsule null locus (cnl), a strain lacking the capsule, demonstrating that LPS core recognition is not influenced by capsule (Figure S2B). Taken together, the immunolabeling studies with postimmune sera of the Alum group raised with tetrasaccharide 1 revealed broad binding to various viable N. meningitidis strains.

**Binding of Anti-tetrasaccharide 1 Antibodies to LPS Inner Core of Closely Related Gram-Negative Bacteria**

Since most Gram-negative bacteria express LPSs that contain core structure motifs shared with tetrasaccharide 1, we determined the specificity of antibody recognition by postimmune sera to a panel of Gram-negative bacteria, while LPS-free Gram-positive bacteria served as negative controls (Figure 6B). No cross-reactivity was observed for closely related N. mucosa or other Gram-negative and Gram-positive bacteria. Significant cross-reactivity only occurred to N. lactamica. This finding is in accordance with earlier reports wherein mAb B5 generated against ΔαgalE LPS also cross-reacted with N. lactamica (Plested et al., 1999). It was shown that the LPS of N. lactamica contains antigens cross-reacting to meningococcal immunotypes L3, L7, and L9 (Braun et al., 2004). These results indicate the high specificity of the anti-tetrasaccharide 1 antibodies to N. meningitidis.

**Immunoprotective Effects of Anti-tetrasaccharide 1 Antibodies**

Besides their binding to the pathogen, functional antibodies are required to eliminate bacteria and protect from infection. Two in vitro assays are currently used to assess the functional activity of antibodies: the opsonophagocytic activity (OPA) (Ja¨kel et al., 2001). For the SBA measurement of N. meningitidis B, a human complement source, typically serum, should be used because increased bactericidal titers were observed in the presence of rabbit complement (Santos et al., 2008; Plested et al., 2001) and SBA assays. The gold standard test to detect N. meningitidis lysis in the presence of complement is the SBA assay that correlates with immunoprotection (Plested et al., 2001). For the SBA measurement of N. meningitidis B, a human complement source, typically serum, should be used because increased bactericidal titers were observed in the presence of rabbit complement (Santos et al., 2001; Zollinger and Mandrell, 1983). In contrast, SBA of N. meningitidis serogroup C can be conducted with rabbit complement source. Thus, we performed our SBA assay with N. meningitidis serogroup C expressing homologous immunotype L3 wt (WUE2120) and the corresponding truncated Δpgm mutant (WUE2420). Despite the broad and specific recognition of N. meningitidis strains by serum antibodies, no serum-dependent bactericidal activity was detected (Figures S3A–S3C). The OPA measures antibody opsonization of N. meningitidis that triggers uptake into phagocytic cells such as neutrophils, resulting in protection against bacteremia. The postimmune sera of the Alum group showed a tendency toward higher OPA titers for MC58 wt (p = 0.08; Figure S3D).
Further analysis of tetrasaccharide 1-specific serum antibodies revealed IgG1 antibodies in mice (Burgener et al., 2006). Isotypes IgG3 and IgG2a are described to promote SBA and OPA (Michaelsen et al., 2004). The absence of isotype IgG3 and the low amount of isotype IgG2a may explain the lack of SBA and low OPA. Improved formulation strategies using approved Th1-skewing adjuvants that force a class switch toward IgG2a and IgG3 may elicit protective antibodies against *N. meningitidis* infection (Baudner et al., 2009; Lin et al., 2004).

**Determination of Conserved Immunogenic Epitope of Anti-LPS Inner Core Antibodies**

Although we observed broad binding of the serum antibodies to viable *N. meningitidis* strains, the absence of functional antibodies suggests that presentation of the LPS core requires improvement. We aimed to determine the essential structural requirements of the LPS core antigen that may elicit protection at the molecular level, we generated tetrasaccharide 1-specific mAb from a mouse via hybridoma technique (Anish et al., 2013; Köhler and Milstein, 1975). Two mAbs, termed 1A5 1G1 and 1B6 4E1, were purified to homogeneity (Figure S6A). In glycan array analysis, mAb 1A5 1G1 recognized all oligosaccharides interacting with polyclonal sera except trisaccharide 1 (Figures S5C and 7D). mAb 1A5 1G1 has a binding preference for Kdo-containing epitopes (Figures 3C and 7D). For mAb 1A5 1G1, dissociation constant (K_D) values in the low micromolar range were observed (Figures S6F and S6G). For mAb 1A5 1G1, dissociation constant (K_D) values in the low micromolar range were observed (Figures S6F and S6G). No
concentration-dependent binding was observed for trisaccharide 2 and α-Kdo 4, disclosing selective binding of 1B6 4E1 to tetrasaccharide 1 (data not shown). The reactivity of these mAbs suggests that immunization with tetrasaccharide 1 elicited antibodies strongly binding to Kdo-containing structures with insignificant binding to the crucial distal trisaccharide 5. In Figure 6. Immunolabeling of Viable N. meningitidis LPS Core Mutants, and Gram-Negative and Gram-Positive Bacteria, with Tetrasaccharide 1-Specific Mouse Sera of the Alum Group
(A) Binding of postimmune sera of the Alum group and control group to viable N. meningitidis (immunotype L3) MCS8 mutants expressing truncated LPS measured by ELISA-based assay.
(B) Binding of postimmune sera of the Alum and control groups to viable related Neisseria species, Gram-negative and Gram-positive bacteria measured by an ELISA-based assay. Detection of bound antibodies was performed with secondary anti-mouse IgG-HRPO antibody. Fold change in mean optical density 450 nm of the Alum group, with respect to control group, is plotted. Average of three independent experiments with mean of three replicates is shown. Error bars represent SEM. *p < 0.05, two-sided unpaired t tests against ΔlpxA.
contrast to the postimmune sera, both mAbs did not recognize the LPS core on viable *N. meningitidis* strains. The broad cross-reactivity to synthetic LPS cores observed on the glycan arrays may be caused by the presentation of Kdo residues via their pentyl amino linker on the array surface. On the contrary, accessibility to Kdo may be restricted on natural bacterial surfaces due to close proximity to the outer membrane. Moreover, postimmune serum antibodies bound to compound 5, whereas the mAbs did not (Figure 7). Thus, exposure of distal trisaccharide 5 on the surface of *N. meningitidis* most likely permits binding of postimmune sera to the LPS core. In another immunization approach, BSA conjugates with different antigen loadings of the synthetic oligosaccharides from *Vibrio cholerae* O1 Inaba O-polysaccharide elicited a comparable level of antibodies with limited protection (Meeks et al., 2004).

To further refine the structural requirements for LPS recognition by antibodies, we tested mAb B5 raised against LPS of ΔgalE mutant (a kind gift from Dr. A.D. Cox). mAb B5 showed SBA and reacted against several *N. meningitidis* strains expressing LPS with PEtN at 3-position of HepII (Plested et al., 1999, 2003). In a glycan array screening, mAb B5 did not bind to any synthetic LPS core, probably due to the absence of a PEtN modification (Figure S7A). To further delineate the immunodominance of Kdo, we tested the binding of our antibodies against isolated and delipidated ΔgalE LPS that was immobilized on an array slide via an adipic acid dihydrazide (ADH) spacer. This immobilization destroyed the α-Kdo motif due to derivatization at its anomeric position by hydrazide group with predominant β-configuration (Lee and Shin, 2003). mAb B5 bound to immobilized ΔgalE LPS core, whereas our mAbs did not (Figures S7B and S7C). mAb B5 binds to the LPS core via the trisaccharide 5 derivative with an additional PEtN on HepII and Glc on Hep (Plested et al., 1999), independent of the α-Kdo motif. While postimmune sera of the FA group showed weak reactivity to immobilized ΔgalE LPS core compared to mAb B5, postimmune sera from the Alum group did not show any binding to ΔgalE LPS core (Figures S7B and S7C). This effect may be explained by higher avidity of tetrasaccharide 1-specific antibodies raised by the Th1-skewing FA compared to Th2-skewing Alum (Figures S4B and S4C). Accordingly, higher signal intensities were gained for FA sera compared to Alum in glycan array screening on synthetic LPS core structures (Figures 7A and 7B).

Based on glycan array screening, SPR, and bacterial immunolabeling, we infer that the trisaccharide motif represented by 5 is the crucial component of an LPS core antigen against *N. meningitidis*. This distal LPS core 5 was recognized by postimmune sera produced against synthetic tetrasaccharide 1, but antibody levels were low compared to that raised against Kdo-containing structures 1–4 and 8–10. Moreover, human sera predominantly recognize structure 5 (Figure 3). Furthermore, bacte- ricidal mAb B5 bound only weakly to the *N. meningitidis* ΔicsB mutant expressing tetrasaccharide 1 derivative with an additional PEtN on HepII (identical phenotype to Δpgm) (Plested et al., 1999), substantiating the hypothesis that distal parts of the LPS

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**Figure 7. Specificity of Mouse Sera of the Alum and FA Groups and of Tetrasaccharide 1-Specific mAbs 1A5 1G1 and 1B6 4E1 toward 12 Synthetic LPS Core Structures Inferred by Glycan Array**

(A–D) Representative array scan of sera from the Alum group (A, left) of mAbs 1A5 1G1 (C, left) and 1B6 4E1 (C, right) and the printing pattern (A, right) are depicted. Quantification of mean fluorescence intensity of postimmune sera (B) and mAbs (D) are also shown. Error bars represent SD of two spots of two separate arrays, respectively.

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Chemistry & Biology
Defined LPS Inner Core Antigen for *N. meningitidis*

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core are important in eliciting SBA. Due to the predominant reactivity of mAb B5 against PETN modification on HepII, it only bound to 76% of N. meningitidis B strains expressing this specific PETN but not to strains with alternative modifications (Plested et al., 1999). Avoidance of LPS core variants such as PEtN modification and Gic residue on HepII may recruit more broadly reactive antibodies. Thus, by leaving out Kdo, the highly conserved LPS core trisaccharide might already be sufficient to raise a broadly protective immune response. Furthermore, via addition of conserved Gic to Hep in 5, an enlarged, branched, and more surface-exposed LPS core \( \alpha-D-GlcNAC-(1 \rightarrow 2)-L-\alpha-D-Hep-(1 \rightarrow 3)-[\beta-D-Glc-(1 \rightarrow 4)]-L-\alpha-D-Hep \) is formed that may elicit both broad cross-reactivity to N. meningitidis and bactericidal activity. This assumption is substantiated by the broadly cross-protective mAb WN1 222-5, which binds to the LPS inner cores of E. coli and Salmonella. The 4-phosphate groups on the branched HepII and the HepIII side chains were identified as the minimal structural determinants for high-affinity binding to these LPS inner core structures. This finding demonstrates the relevance of branched structures (Müller-Loennies et al., 2007).

Currently, efforts are underway to redesign the synthetic antigen to generate adequate antibody levels to distal parts of the N. meningitidis LPS inner core with functional activity. Here, we have shown the power of synthetic LPS core oligosaccharides in defining a universal antigen candidate for N. meningitidis. A T cell-dependent antibody response specific to conserved LPS inner core was elicited in mice. The immunodominant \( \alpha-Kdo \) residue accounted for the lack of functional antibodies. In contrast, the distal trisaccharide mediates specific recognition of the natural LPS core on N. meningitidis. Based on our findings, an improved synthetic LPS core compared to compound 5 is currently being explored as a broadly protective antigen against N. meningitidis.

**SIGNIFICANCE**

Designing a synthetic carbohydrate antigen candidate involves the choice of the optimal structure to confer protection. Today, antigen identification is an iterative process involving trial-and-error experiments. Here, we report progress in this process using a highly conserved LPS core structure of N. meningitidis as an example. Antigenicity and immunogenicity of synthetic LPS core oligosaccharides were evaluated using glycan arrays, in vivo immunogenicity studies, SPR, and in vitro surrogate assays for functional bacteria-killing antibodies. In combination with the epitope mapping of earlier described protective mAb B5, structural information of a potential, broadly reactive LPS core epitope was inferred. Glycan array screening of patient sera revealed antibodies specific to different LPS inner core structures. Based on these screenings, a tetrasaccharide was selected for immunological evaluation in mice and, therefore, conjugated to carrier protein CRM197. This glycoconjugate yielded LPS core-specific robust IgG and memory responses. Serum antibodies bound to numerous viable N. meningitidis strains. The immunodominant Kdo moiety contained in this tetrasaccharide elicited highly affine antibodies against Kdo-containing oligosaccharide but failed to bind to N. meningitidis bacteria. We identified the distal part of the LPS core as the crucial unit to recruit antibodies that are broadly reactive to N. meningitidis expressing diverse LPS immunotypes. Based on our results, we propose an improved, highly conserved LPS core tetrasaccharide \( \alpha-D-GlcNAC-(1 \rightarrow 2)-L-\alpha-D-Hep-(1 \rightarrow 3)-[\beta-D-Glc-(1 \rightarrow 4)]-L-\alpha-D-Hep \) to generate antibodies that may unify broad reactivity and bactericidal activity against N. meningitidis.

**EXPERIMENTAL PROCEDURES**

**Chemical Synthesis**

A detailed description of experimental procedures and characterization of synthetic compounds are available in Supplemental Experimental Procedures.

**Human Sera**

Convalescent sera used have been published recently (Hubert et al., 2013). Sera from healthy individuals carrying or not carrying N. meningitidis were obtained from human volunteers, with permission of the Ethics Committee of the Medical Faculty of the Julius-Maximilians-University.

**Ethics Statement**

Animal experiments were performed in accordance with the German regulations of the Society for Laboratory Animal Science and the European Health Law of the Federation of Laboratory Animal Science Associations. All efforts were made to minimize the suffering of the animals.

**Immunization Studies and Evaluation of Immune Response Using SPR and Microarray**

Glycoconjugate of CRM197 and amino-linked antigen was produced, characterized, and immunized in BALB/c mice in a dose- and formulation-dependent manner (Martin et al., 2013). Splenocytes of a mouse with the highest antibody titer (FA group) were used to generate mAbs via the hybridoma technique (Anish et al., 2013; Köhler and Milstein, 1975). Specificities of serum antibodies and purified mAbs were analyzed by glycan array screening. A glycan array of synthetic compounds was prepared, and antibody samples were incubated as described elsewhere (Martin et al., 2013). For immobilization of purified glycans, slides were modified as reported elsewhere (Lee and Shin, 2009). Affinity of direct immobilized mAbs to solubilized glycans and binding of mouse sera to immobilized glycans was analyzed by SPR.

**Immunolabeling Studies of Bacteria and Functional Activity Test**

Staining of bacteria with mAb and polyclonal sera was performed as described elsewhere (Anish et al., 2013). Antibodies bound to viable bacteria were detected via secondary goat anti-mouse IgG (H+L)-HRPO antibody, and readout was accomplished at 450 nm in a microplate reader. Antibodies bound to inactivated bacteria were detected by appropriate fluorescent-labeled secondary antibody and measured by flow cytometry or confocal fluorescence microscopy. SBA (Elías et al., 2013) and OPA (Hazenberg et al., 1994; Rodríguez et al., 2001) of sera were tested as described elsewhere. For a detailed description, please refer to the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information including Supplemental Experimental Procedures, seven figures, and one table can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.11.016.

**AUTHOR CONTRIBUTIONS**

A.R. performed animal studies, generation of mAbs, immunolabeling, and functional activity assays. Y.Y. synthesized LPS core structures with the help of C.L.P., who also initiated the LPS core screening project. A.D.C. provided mAb B5 and detoxified LPS core structure. H.C. performed mutagenesis, bacterial culture, and initial immunolabeling experiments with the help of U.V.C.A. and P.H.S. designed and initiated this study. A.R., C.A., and P.H.S. wrote the manuscript with input from all authors.
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