Lipo-oligosaccharides (LOS) produced by Neisseria gonorrhoeae are important antigenic and immunogenic components of the outer membrane complex. Previously, we showed that murine monoclonal antibody (mAb) 2C7 did not cross-react with human glycosphingolipids but identified the LOS epitope that is widely expressed in vivo and in vitro (Gulati, S., McQuillen, D. P., Mandrell, R. E., Jani, D. B., and Rice, P. A. (1996) J. Infect. Dis. 174, 1223–1237). In the present study, we analyzed the structure of gonococcal strain WG LOS containing the 2C7 epitope and investigated the structural requirements for expression of the epitope. We determined that the WG LOS components are Hep[1]-elongated forms of 15253 LOS that have a lactose on both Hep[1] and Hep[2] (Yamasaki, R., Kerwood, D. E., Schneider, H., Quinn, K. P., Griffiss, J. M., and Mandrell, R. E. (1994) J. Biol. Chem. 269, 30345–30351). In addition, we found that expression of the 2C7 epitope within the LOS is blocked when the Hep[2]-lactose is elongated. Based on the structural data of these LOS and the results obtained from immunochemical analyses, we conclude the following: 1) mAb 2C7 requires both the 15253 OS minimum structure and the N-linked fatty acids in the lipoidal moiety for expression of the epitope; 2) mAb 2C7 binds to the LOS that elongates the lactose on Hep[1] of the 15253 OS, but not the one on Hep[2]; and 3) the 2C7 epitope is expressed on gonococcal LOS despite the presence of human carbohydrate epitopes such as a lactosamine or its N-acetylgalactosaminylated (globo) form. Our study shows that the conserved epitope defined by mAb 2C7 could potentially be used as a safe site for the development of a vaccine candidate.

Neisseria gonorrhoeae causes one of the major sexually transmitted human diseases. The potential severity of complications of gonococcal infection has provided impetus to develop a preventive anti-gonococcal vaccine. In response to natural gonococcal infection, the antibody response is primarily directed against pili, outer membrane proteins, and lipo-oligosaccharide (LOS)1 (1, 2). Prior vaccine attempts using gonococcal pilus, porin, and opacity protein have failed to produce a broadly protective immune response (3–5). Therefore, several investigators have been concentrating efforts to utilize LOS as potential vaccines.

Recent immunochemical and structural studies have shown that the oligosaccharide (OS) structure governs complex antigenic variations among gonococcal LOS (6–14). The OS moiety consists of a conserved core and a structurally variable region. Gonococci synthesize this variable region by adding a glycosyl moiety sequentially on the conserved core trisaccharide, GlcNAc-Hep[2]-Hep[1]2 to express two different types of OS elongation; OS elongates from Hep[1] only or from both Hep[1] and Hep[2]. Different OS structures thus produced result in heterogeneous antigenic expression of LOS whose molecular masses vary in the range of 3–7 kDa (6).

In addition to complex LOS expression, studies by us and others (8, 11) have shown that gonococci may mimic human glycolipids or may change serum sensitivity by modifying their LOS (15–17). The 4.5-kDa LOS produced by strain F62 contains a lactonetetraose moiety that is linked to the Hep[1] residue of the core trisaccharide as described above (11). This tetraose is the carbohydrate moiety also found in a human glycosphingolipid, paragloboside (8, 11). Furthermore, in vitro experiments showed that gonococci utilize exogenous cytidine monophospho-N-acetylneuraminic acid and sialylate their LOS (17). As a result of sialylation, gonococci become resistant to complement-mediated killing (15, 16). Thus, gonococci may evade human immune defenses by mimicking human carbohydrate epitopes or altering their serum sensitivities.

1 The abbreviations used are: LOS, lipo-oligosaccharide(s); COSY, chemical shift correlation spectroscopy; DQF, double quantum-filtered; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray ion mass spectrometry; FAB-MS, fast atomic bombardment mass spectrometry; Hep, heptose; Hex, hexose; HexNac, N-acetylhexosamine; HF, hydrofluoric acid; HO-HAHA, homonuclear Hartman-Hahn spectroscopy; KDO, 2-keto-3-deoxy-manno-octulosonic acid; mAb, monoclonal antibody; MS, mass spectrometry; NOE, nuclear Overhauser effect; OS, oligosaccharide(s); PEA, phosphoethanolamine; PAGE, polyacrylamide gel electrophoresis.

2 To distinguish the two heptoses in the core OS region, we defined the Hep linked to KDO as Hep[1] and the other Hep linked to Hep[1] as Hep[2].

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Despite the frequency of antigenic heterogeneity of gonococcal LOS often accompanied by cross-reaction with human glycosphingolipids or gangliosides, our previous studies (18) had indicated the possibility to overcome the potential problems inherent in the variability of gonococcal LOS in order to develop LOS-based vaccines. Our recent studies showed that murine mAbs, 3G9 and 2C7, do not cross-react with human glycosphingolipids or gangliosides (18). Of the epitopes defined by these mAbs, the 2C7 epitope is widely expressed in vivo and in vitro (7, 18). In addition, mAb 2C7 is bactericidal and opsonic against gonococci converted to serum resistance by sialylation or strains that are otherwise intrinsically serum-resistant (18). Further study with mAb 2C7 showed that its anti-idiotypic antibody elicited a bactericidal and opsonic immune response in experimental immunization (19). Collectively, these data indicated that mAb 2C7-defined epitope could potentially be utilized as a safe vaccine target.

Both previous and very recent work have provided some insights on the mAb 2C7-defined epitope. First, mAb 2C7 did not bind to the lipid A moiety of the LOS but to its tyraminated OS, which suggested that the mAb recognizes the OS moiety of the LOS (18). Second, inert binding of the mAb to some LOS samples has shown that LOS lacking OS on the Hep[2] may not have the 2C7 epitope (18, 20). Third, immunoblot analysis using truncated gonococcal LOS has suggested the importance of a lactose on Hep[2] for expression of 2C7 epitope (21). However, those previous studies have not yet precisely confirmed whether mAb 2C7 recognizes gonococcal LOS of a specific OS elongation pattern. Neither the 2C7 epitope nor the structural requirements necessary for its expression have been defined.

Among LOS recognized by the mAb 2C7 (7, 18, 21), LOS produced by an isolate from disseminated gonococcal infection (DGI), 15253, is the only one whose OS structure has been determined (13). Definition of LOS structure other than 15253 LOS is essential for us to determine not only the specific OS elongation pattern that mAb 2C7 recognizes but also the common epitope expressed among various gonococcal LOS. Therefore, in the present study, we studied the structures of LOS components produced by another DGI isolate, WG (22). In addition to the structural analysis, we investigated structural requirements for the binding of mAb 2C7 to several additional gonococcal LOS thereby characterizing the 2C7 epitope. We determined that the major WG LOS contains a Galβ1-4GlcNAcβ1-3Galβ1-4Glc (lactoneotetraose) and a Galβ1-4Glc (lactose), respectively, on Hep[1] and Hep[2] of the GlcNAc-Hep[2]-Hep[1] trisaccharide core. mAb 2C7 recognizes the inner 15253 LOS present in WG LOS as a minimum structure and requires N-linked fatty acids in the lipid A for maximum expression of its epitope. In addition, mAb 2C7 binds to LOS whose OS are elongated from the lactose on Hep[1] of the 15253 OS but not on Hep[2], and the 2C7 epitope is expressed on gonococcal LOS despite the presence of human carbohydrate epitopes such as lactosamine or its N-acetylgalactosaminylated (glob) form.

MATERIALS AND METHODS

**LOS and mAbs**—We used the following gonococcal LOS that have been prepared previously: F62 (11), M511mk (variant A) (12), WG (22), 15253 (13), 24-1 (18). 15253 lgtE mutant LOS was provided by Dr. Emil C. Gotschlich (The Rockefeller University, New York), and JW31R LOS was provided by Dr. Michael A. Apicella (University of Iowa, Iowa City) and Dr. Robert E. Mandrell (USDA/ARS, Albany, respectively). The production of mAb 2C7 (7, 18) has been described previously.

**LOS Modification and PAGE Blot Analysis**—Partial deacetylation with NaOH and dephosphorylation of LOS were done as described previously (10, 12, 24). LOS samples were also de-O-acylated with anhydrous hydrzone by modifying the method by Haishima et al. (25); LOS were treated in anhydrous hydrzone at room temperature (−25 °C) for 2 h. The resulting LOS were further treated with 4 M KOH for 4 h at 120°C for complete deacetylation (25, 26). Enzymatic treatment of WG, 15253, and JW31R LOS were done as described previously (10). β-N-Acetylgalactosaminidase (from jack beans) and β-galactosidase (from Aspergillus niger) were purchased from Sigma.

**PAGE Blot and ELISA**—Intact LOS and chemically and enzymatically modified LOS were separated with a Bio-Rad mini-PROTEAN 2 Cell (Bio-Rad), and blots were immunostained with subsequent treatments of mAbs, goat anti-mouse IgG, or IgM Abs (alkaline phosphatase conjugated) and Western Blotting Stabilized Substrate (Promega Co. Madison) (10, 12, 24). TLC immunostaining of LOS was done as described previously (10) by using anti-mouse IgG (alkaline phosphatase conjugate) and then with a substrate solution (p-nitrophenolate). Absorbancies at 405 nm were measured with a Multiskan MS (Labsystems, Helsinki, Finland). After determining the concentration of mAb 2C7 for ~50% binding to the intact 15253 LOS, the binding of the mAb to the LOS (100 ng) was measured in the presence of inhibitors (25–800 ng). The secondary antibodies used in this study were purchased from Sigma.

**Oligosaccharide (OS) and Carbohydrate Analyses**—Preparation of OS, hydrolysis, and carbohydrate analyses were done as described in previous studies (10–13, 27) unless otherwise stated. A major OS component (dephosphorylated) was obtained after gel chromatography (a Bio-Gel P-6 column, <400 mesh, 1.6 × 90 cm, 100 mM ammonium acetate) and subsequent desalting with Bio-Gel P-2 chromatography. For gas chromatography-MS analysis, a JEOL AX505HIA spectrometer was used. The following partially methylated alditol acetates were identified in the major WG OS component: 2,3,4,6-tetra-O-Me-Gal; 2,3,6-tri-O-Me-Gal; 2,4,6-tri-O-Me-Gal; 3,6-di-O-Me-GlcNMeAc; 3,4,6-tri-O-Me-GlcNMeAc; 2,6,7,7-tri-O-Me-Hep; and 4,6,7-tri-O-Me-Hep. The partially methylated alditol acetates gave identical fragmentation patterns to those as reported previously (11–13, 27).

**Mass Spectral Analyses**—Negative ESI (electrospray ionization) mass spectra of de-O-acylated WG LOS were obtained with a triple stage quadrupole mass spectrometer (Finnigan MAT TSQ 700) equipped with the ESI ion source (28). Samples were dissolved in water/acetonitrile (1:1 (v/v)) containing 0.1% ammonia solution. The ESI mass spectrum conditions are as follows: potential difference in the negative ion mode, 3 kV; capillary temperature, 200 °C; sample flow rate, 5 ml/min; sheath and auxiliary gas, nitrogen; sheath gas pressure, 60 mtorr; and collision gas pressure, 0.4 Pa. The data were processed with a DEC Station 5000/120 computer. The FAB (fast atom bombardment)-mass spectra of oligosaccharides (OSs) were obtained with the Finnigan MAT TSQ 700 mass spectrometer equipped with the FAB ion source (29, 30). Samples were dissolved in water. The FAB mass spectra were recorded under the following conditions: primary beam, xenon; accelerating voltage of the primary ion, 8 kV; collision gas, argon; collision energy, 30 eV; collision gas pressure, 0.4 Pa. The data were processed with a DEC Station 2100 computer.

**NMR Analysis**—All NMR experiments were run on a JEOL 600 MHz spectrometer in D₂O at 25 °C. Two-dimensional NMR spectra were obtained in a similar manner as described (11–13, 31). DQF-COSY (number of acquisition = 32, sweep width = ±2000 Hz, the 1 × 4K data points were processed to give the final 2 × 4K points, and digital resolutions were 0.68 and 1.37 Hz/point in the ω1 and ω2 axes, respectively), HOHAHA (number of acquisition = 64, sweep width = ±2000 Hz, Tm = 120 ms, the 0.5 × 2K data points were processed to give the final 1 × 4K points); two-dimensional NOE (number of acquisition = 96, sweep width = ±2000 Hz, Tms = 200 ms, the 0.5 × 2K data points were processed to give the final 1 × 4K points).

**RESULTS AND DISCUSSION**

From a panel of LOS recognized by mAb 2C7, we selected a DGI isolate, WG. This strain produces two LOS components whose molecular weights are higher than 15253 LOS, and mAb 2C7 binds to both (Fig. 1). To obtain the molecular mass and preliminary composition of the WG LOS, we analyzed the O-de-acylated WG LOS by negative electrospray ion MS (ESI-MS). Treatment of the LOS with anhydrous hydrzone at room temperature (−25 °C) for 2 h gave a water-soluble de-O-acylated
**Analysis of Structure and Epitope of LOS Produced by N. gonorrhoeae**

LOS preparation. This ESI-MS analysis indicated that the major O-deacylated WLGS has the following general structure: \((\text{Hex})_5-(\text{HexNAc})_2-(\text{Hep})_2-(\text{KDO})_2-(\text{de-O-acylated lipid A})\) with \((\text{PEA})_{0–2}\).

This composition was indicated by the presence of three sets of triply and doubly charged ions of the LOS and singly charged ions of its OS (Fig. 2 and Table I); for example, the most abundant triply charged ion at \(m/z\) 1558.1 and a doubly charged ion at \(m/z\) 1533.1, that correspond to the composition of \((\text{Hex})_5-(\text{HexNAc})_2-(\text{Hep})_2-(\text{PEA})_{0–2}\)-acylated lipid A. Its OS counterpart (singly charged) is seen in Fig. 2 as a dehydrated form at \(m/z\) 2163.7. The mass differences between the OS-de-acylated lipid A with \((\text{PEA})_{0–1}\) and their OS (Table I) confirmed that the intact LOS was de-O-acylated with the hydrazine treatment described above.

Out of singly charged ions, the mass of 1820.3 corresponding to \((\text{Hex})_5-(\text{HexNAc})_2-(\text{Hep})_2-(\text{KDO})_2\) (Fig. 2) was also detected in ESI/MS-MS (data not shown) of the doubly charged ion at \(m/z\) 1496.3 of Fig. 2. This ESI/MS-MS analysis confirmed that the ion at \(m/z\) 1820.3 is due to the OS moiety. In addition, the peak at \(m/z\) 2146.8 in Fig. 2 suggested the presence of the OS derived from a minor LOS component, an adduct of HexNAc to \((\text{Hex})_5-(\text{HexNAc})_2-(\text{Hep})_2-(\text{PEA})\), whose identity will be described later. The molecular compositions of the major WLGS predicted with the above ESI-MS was also confirmed by FAB-MS and compositional analysis described below.

**FAB-MS analysis** (Fig. 3) of a major WLGS (dephosphorylated), obtained after P-6 gel chromatography, indicated the presence of the ion at \(m/z\) 1837.9 and its dehydrated form (at \(m/z\) 1820.2), this molecular ion at \(m/z\) 1837.9 corresponds to \((\text{Hex})_5-(\text{HexNAc})_2-(\text{Hep})_2-(\text{KDO})_2\), and this result was consistent with the presence of the singly charged ion at \(m/z\) 1820.3 in the ESI/MS analysis (Fig. 2 and Table I). The FAB-MS spectrum also indicated the following sequence ions together with their hydrated forms: 1675.7 (\(-\text{Hex}\)), 1472.6 (\(-\text{Hex-HexNAc}\)), 1310.6 (\(-\text{Hex-HexNAc-Hex}\)), and 1148.0 (\(-\text{Hex-HexNAc-Hex-Hex}\)).

**Compositional analysis** of the major OS showed the molar ratio of Gal/Glc/GlcNAc/Hep/KDO is 2.8:2.1:1.7:1.8:1, which showed that the anomeric proton of GlcNAc linked to Gal(IX) was determined by the inter-residual NOE (Fig. 6) confirmed that the residue V is GlcNAc but not Glc. We determined that the anomeric proton (VI) at 4.56 ppm is Glc but not GlcNAc based upon the methylation data. The presence of a terminal GlcNAc in the methylation analysis does not support VI to be GlcNAc, and a terminal Glc should have been present in the methylation analysis if the residue III were Glc and not GlcNAc. The lactosamine, Galβ1-4GlcNAcb1-3Galβ1-4Glc is on Hep(VI-IV). We also determined the following: 1) Gal(VIII)-Glc(II) and GlcNAc(III), respectively, are α1-3- and α1-2-linked to Hep(I), and 2) this Hep(I) is α1-3-linked to Hep(IV). Methylolation data (terminal Gal, 4-linked Glc, 2,3-di-linked Hep, and terminal GlcNAc) and the inter-residual NOEs due to VIII-I-II-IV, II-1/3, and III-1-2 confirmed the structure. The H-3 and H-4 of Gal (VIII) were almost identical to those of the corresponding Gal residue of 15253 OS (12), which confirmed that the lactosamine described above is not linked to the Gal (VIII).

These structural data show that strain WG produces elongated forms of 15253 LOS that has a lactose on both Hep[1] and Hep[2] (13). Stain WG elongates only the lactose on Hep[1] of 15253 LOS to express human carbohydrate epitopes, a lactosamine and its N-acetylglactosaminylated form (globo) at the non-reducing end. Our structural study revealed that mAb...
2C7 binds to the 15253 LOS structure contained within the two WG LOS components despite the presence of those human carbohydrate epitopes. After determining the structures of the WG LOS components, we analyzed the 2C7 epitope. First, we clarified that mAb 2C7 recognizes a specific carbohydrate elongation pattern, OS on both Hep[1] and Hep[2] as represented by 15253 and WG LOS. Second, by analyzing epitope expression of another gonococcal LOS whose carbohydrate elongates from the lactose on Hep[2] of 15253 LOS, we further specified the OS elongation pattern recognized by mAb 2C7. Third, we defined the basic OS structure necessary for expression of the 2C7 epitope. Fourth, we characterized structural requirements other than the OS structure for the maximum expression of the 2C7 epitope.

In order to clarify the specific OS elongation pattern recognized by mAb 2C7, we analyzed a series of LOS having OS only on Hep[1] of the core Hep[1]-Hep[2] diheptose for their binding capability to mAb 2C7. For this purpose, we used previously characterized gonococcal LOS, F62 (11) and MS11mk (variant A) (12), together with their truncated forms that had been prepared previously (10, 12). The results obtained by ELISA

![Image](https://example.com/image1.png)

**Fig. 2.** Negative ESI-MS analysis of O-deacylated WG LOS. Calculated masses were based on isotopic ions: CH₃COOH, 42.0105647; PEA, 141.019097; Hex, 162.063388; HexNAc, 203.079372, KDO, 220.058303. We used 952.467406 for the mass number for the de-O-acylated lipid A (34).

**TABLE I**

| Observed $M_r$ | Calculated $M_r$ | Proposed composition |
|----------------|-----------------|----------------------|
| 3240.9 (1079.3, 1619.1) | 3241.2 (1079.4, 1619.6) | (Hex)$_5$-(HexNAc)$_2$-(Hep)$_2$-(KDO)$_2$-(PEA)$_2$-(de-O-acylated lipid A) |
| 3118.2 (1038.1, 1558.1) | 3118.2 (1038.4, 1558.1) | (Hex)$_5$-(HexNAc)$_2$-(Hep)$_2$-(KDO)$_2$-(PEA)$_2$-(de-O-acylated lipid A) |
| 2994.6 (997.2, 1496.3) | 2995.1 (997.4, 1496.6) | (Hex)$_5$-(HexNAc)$_2$-(Hep)$_2$-(KDO)$_2$-(de-O-acylated lipid A) |
| 2775.4 (1386.7) | 2775.1 (1386.5) | (Hex)$_5$-(HexNAc)$_2$-(Hep)$_2$-KDO-(de-O-acylated lipid A) |
| 2182.7 (2163.7) | 2181.7 (2162.7) | (Hex)$_5$-(HexNAc)$_2$-(Hep)$_2$-PEA |
| 2059.4 (2040.4) | 2058.7 (2039.7) | (Hex)$_5$-(HexNAc)$_2$-(Hep)$_2$-KDO |
| 1962.3 (1943.3) | 1961.6 (1942.6) | (Hex)$_5$-(HexNAc)$_2$-(Hep)$_2$-KDO-PEA |
| 1839.3 (1820.3) | 1838.6 (1819.6) | (Hex)$_5$-(HexNAc)$_2$-(Hep)$_2$-KDO |

* Triply charged ion.
* Doubly charged ion.
* Singly charged ion (as a dehydrated form).

![Image](https://example.com/image2.png)

**Fig. 3.** FAB-MS analysis of the major WG OS (dephosphorylated). Triethanolamine (TEA) was used as the matrix in the negative ion mode.

![Image](https://example.com/image3.png)

**Fig. 4.** A part of one-dimensional NMR spectrum (600 MHz at 25 °C, resolution enhanced). The roman numerals refer to the nine different carbohydrate residues as shown in the structure.
and PAGE blot analyses are summarized in Table III. mAb 2C7 bound none of the gonococcal LOS tested, which confirmed partial results obtained by us (18) and by Banerjee et al. (21). In addition, we re-examined the binding of mAb 2C7 to 24-1 LOS. Previous studies (18) showed that mAb 2C7 bound to 24-1 LOS, although carbohydrate elongation of the major 24-1 OS components were found to occur only on Hep[1] (20). We were not be able to detect mAb 2C7 binding to 24-1 LOS at the usual testing level of 100 ng by Western blot; 250 ng of the LOS was necessary for the detection of a minor component whose PAGE mobility was almost identical to 15253 LOS (data not shown). The present analysis indicated that the previous positive binding of mAb 2C7 to 24-1 LOS is due to the presence of a trace amount of LOS whose OS structure is presumably to be same as that of 15253 LOS. Thus, based upon the above results obtained, we concluded that the 2C7 epitope resides on gonococcal LOS that contain OS on both Hep[1] and Hep[2] and that the LOS containing OS only on Hep[1] do not display the 2C7 epitope.

Then, we further defined the specific OS pattern that mAb 2C7 recognizes and found that the 2C7 epitope is not expressed when the lactose on Hep[2] of 15253 LOS is elongated. As Fig. 8 shows, mAb 2C7 did not bind to JW31R LOS (9) but weakly bound to a minor LOS component having the same PAGE mobility as 15253 LOS. However, the mAb 2C7 binding to JW31R LOS increased after it was digested sequentially with β-hexosaminidase and β-galactosidase, which indicated that the major JW31R LOS components are also elongated forms of 15253 LOS. The OS of one of the major JW31R LOS components had been indicated to contain Hex-Hex and GalNAc-Hex-Hex on Hep[1] and Hep[2], respectively (9). This current level of 100 ng by Western blot; 250 ng of the LOS was necessary for the detection of a minor component whose PAGE mobility was almost identical to 15253 LOS (data not shown). The present analysis indicated that the previous positive binding of mAb 2C7 to 24-1 LOS is due to the presence of a trace amount of LOS whose OS structure is presumably to be same as that of 15253 LOS. Thus, based upon the above results obtained, we concluded that the 2C7 epitope resides on gonococcal LOS that contain OS on both Hep[1] and Hep[2] and that the LOS containing OS only on Hep[1] do not display the 2C7 epitope.

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study suggests that Galβ1–4Glc and GalNAc-Gal-Galβ1–4Glc, respectively, are linked to Hep[1] and Hep[2]. The increase of 2C7 binding to JW31R LOS after the β-hexosaminidase treatment is probably due to further cleavage of the enzyme-treated LOS by β-galactosidase present in the β-hexosaminidase preparation since this enzyme preparation of the same unit used for JW31R LOS also cleaved a Gal off the 4.5-kDa F62 LOS component that contain a lactoneotetraose (data not shown). The 2C7 epitope was expressed on WG LOS, the lactose-Hep[1] elongated form, but not on JW31R LOS, the lactose-Hep[2] elongated form of 15253 LOS. This finding shows that mAb 2C7 recognizes only the LOS whose OS elongates from the lactose on Hep[1] of the 15253 LOS and that the 2C7 epitope is not expressed when the lactose on Hep[2] is substituted.

To define the basic OS structure for the 2C7 epitope present within 15253 LOS, we modified 15253 and WG LOS enzymatically and chemically and then analyzed these modified LOS for binding to mAb 2C7. The results are shown in Figs. 9 and 10 and summarized in Table IV. PAGE blot analysis showed that mAb 2C7 bound to the major WG LOS component after the removal of its β-Gal or Galβ1–4GlcNAc from the non-reducing end (Table IV), which confirmed that mAb 2C7 bound to the inner 15253 LOS structure within WG LOS. However, mAb 2C7 did not bind to the β-galactosidase-treated 15253 LOS (marked with an arrow in Fig. 9A, lane 2). The apparent strong binding to the small amount of the intact LOS in lane 2 is due to the fact that a higher concentration of mAb 2C7 was used to distinguish the positive binding from the lack of binding. Although we were not be able to determine which Gal residue was removed from the two lactosyl moieties present in the LOS, this lack of 2C7 binding to the β-galactosidase-treated 15253 LOS is clear evidence that a β-Gal residue at the non-reducing end is indispensable for the epitope expression. The importance of the β-Gal residue was also supported by the fact that mAb did not bind 15253 lgtE mutant LOS (lane 3), that lacks a Gal residue on each lactose attached to Hep[1] and Hep[2] of 15253 LOS (21). These binding studies showed that the β-Gal on the non-reducing end of 15253 LOS is essential for the 2C7 epitope to be expressed. As described above, mAb 2C7 did not bind to JW31R LOS until the lactose on Hep[2] (Fig. 8) was exposed after the
enzymatic treatment. Based upon these results we conclude that mAb 2C7 requires 15253 OS structure as an essential size for its epitope expression.

In addition to the basic 2C7 OS structure, we defined other structural requirements necessary for expression of this carbohydrate epitope by immunochemical analysis of chemically modified LOS samples. We modified both 15253 and WG LOS and obtained almost identical results with those LOS. Fig. 10 shows the results obtained with PAGE blot and TLC immunostain of the modified 15253 LOS samples. mAb 2C7 bound the dephosphorylated LOS obtained with usual treatment with aqueous HF (10). In addition, the mAb bound to the partially deacylated LOS obtained after the NaOH or hydrazine treatment. As described earlier, ESI-MS analysis showed that hydrazine treatment cleaved O-linked fatty acids, and NaOH treatment leads to partial de-N- and O-acylation as studied previously (24). As Fig. 10 shows, the hydrazine treatment of 15253 LOS (lane 4) resulted in heavier aggregation than the NaOH treatment (lane 3), and mAb 2C7 bound to those aggregated LOS as well. This aggregation is probably due to hydrophobic interactions of the N-linked fatty acids remained in the lipid moiety.

Although the partially deacylated LOS samples described above retained the antigenicity, de-N-acylation (4 M KOH for 4 h at 120 °C) (25) of the de-O-acylated LOS resulted in the loss of epitope expression (Fig. 10B, lane 5). Since the completely de-acylated LOS does not adhere to nitrocellulose membrane or ELISA wells, it was analyzed directly in solid phase only by TLC immunoassay (10). The lack of mAb 2C7 binding showed that the presence of N-linked fatty acids is necessary for the maximum expression of the 2C7 epitope and that the N-acetyl group of GlcNAc on Hep[2] may be necessary.

ELISA inhibition studies confirmed the above results obtained by the PAGE blots and TLC immunostain. We analyzed residual mAb 2C7 binding to 15253 LOS in the presence of the chemically modified 15253 LOS samples (25–800 ng) (Fig. 11). The dephosphorylated LOS inhibited the binding as well as the intact LOS. Partially de-acylated 15253 LOS derivatives were slightly less potent inhibitors than the intact LOS. As expected from the TLC blot results (Fig. 10B, lane 5), the completely deacylated LOS did not inhibit mAb 2C7 binding (Fig. 11). However, the 15253 OS that lacks the lipid A was found to be a potent inhibitor compared with the completely deacylated LOS. Since α-N-acetylglucosaminidase is not commercially available, we were not able to digest 15253 LOS with the enzyme to obtain direct evidence for the importance of the GlcNAc. However, the above results obtained by TLC immunostain and ELISA inhibition studies suggested that a GlcNAc moiety on Hep[2] could be also important for the recognition by mAb 2C7.

Taken together, the above immunochemical analyses of chemically modified LOS samples showed 1) phosphate group(s) are not involved in the 2C7 epitope, 2) the O-linked fatty acids are not essential for the 2C7 epitope to be expressed, and 3) in addition to N-linked fatty acids, the GlcNAc moiety linked to Hep[2] could be important for the maximum expression of the 2C7 epitope.

In a previous work, mAb 2C7 was indicated to bind to both 24-1 LOS and its tyraminated OS (18). Very recent mass spectral analysis (20) showed that 24-1 OS elongates only from the Hep[1] of the diheptose. However, 15253 LOS recognized by mAb 2C7 has an OS structure elongating from both Hep[1] and Hep[2]. Therefore, the specific carbohydrate elongation pattern recognized by mAb 2C7 was not clear. In the present study, mAb 2C7 binding to 24-1 LOS was found to be due to the presence of a trace amount of an as yet unidentified LOS component having the same PAGE mobility as 15253 LOS. Also, we found that mAb 2C7 bound to none of the previously characterized gonococcal LOS having carbohydrate elongation only on Hep[1] (Table III). Furthermore, mAb 2C7 did not bind to the major JW31R LOS component (Fig. 8) until the extra disaccharide linked to the lactose on Hep[2] was removed after the sequential enzymatic treatment (Fig. 8). From these results, we conclude that 1) mAb 2C7 binds to 15253 LOS and the LOS whose OS is extended from the lactose on Hep[1] of 15253 LOS and 2) elongation of the lactose on Hep[2] of the 15253 LOS blocks expression of the 2C7 epitope.

Our structural and immunochemical analysis also provided evidence that 15253 LOS contains a basic OS structure necessary for expression of the 2C7 epitope. mAb 2C7 bound to the major WG LOS component after the removal of a galactose or...
lactosamine (data not shown but the results are listed in Table IV). However, it did not bind to 15253 LOS when a β-galactosyl residue was removed from either lactose present at the non-reducing ends (Fig. 9) (Table IV). The necessity of at least one (and perhaps both) of the Gal residues for expression of the 2C7 epitope was supported by the fact that mAb 2C7 bound to the 15253 \textit{igtE} mutant LOS that lacks the Gal moiety of each lactose on Hep[1] and Hep[2] (Table IV). These results do not support the previous suggestion that the lactose on Hep[2] is the 2C7 epitope (21).

In addition to the importance of either Gal moiety of the lactoses, the results obtained from TLC immunoassay and ELISA studies (Figs. 9 and 10) suggested that the GlcNAc on Hep[2] could be important for binding. Direct proof for the importance of the GlcNAc residue could be obtained by producing a 15253 \textit{igtE} mutant lacking the \(\alpha\)-HexNAc residue on Hep[2]. Based upon the results described above, we conclude that mAb 2C7 requires the 15253 LOS structure as a basic structure for its epitope expression.

Besides the 15253 OS structure, some fatty acids present in the lipoidal moiety are important for expression of the 2C7 epitope. Removal of the fatty acids in the lipoidal moiety of 15253 LOS did not affect expression of the 2C7 epitope (Figs. 9 and 10). However, \(\text{N}-\text{acylation} of the de-O-acylated 15253 LOS resulted in the loss of epitope expression (Figs. 9 and 10). This showed that the \(\text{N}-\text{linked} fatty acids in the lipoidal moiety are important for expression of the 2C7 epitope. The 15253 OS structure is defined by the presence of two lactoses linked to the trisaccharide, GlcNAc–Hep[2]–Hep[1], with \(\text{N}-\text{linked} fatty acids present in the lipoidal moiety acting as an anchor to hold the carbohydrate epitope rigid. Removal of the \(\text{N}-\text{linked} fatty acids would lead to the conformational changes of the 15253 OS that defines the 2C7 epitope and, as a result, the loss of full expression of the 2C7 epitope.

Our current structural study of WG LOS also provided evidence that further carbohydrate elongation of the 15253 OS occurs (Fig. 12), which confirmed a previous suggestion (13). Recent studies have identified genes encoding a set of glycosyltransferases for the biosynthesis of the specific OS structures (21, 32, 33). Gonococci add a glycose moiety sequentially on the lactose of Hep[1] and synthesize a lactoneotetraose and its \(\text{N}-\text{acetylgalactosaminylated} form. Our finding of the tetraose on the LOS containing a lactose on Hep[2] evokes the question of how the OS structure represented by WG LOS is synthesized. Gonococci may elongate either the lactose on Hep[1] after the completion of the 15253 OS structure or Hep[2] after the lactoneotetraose on Hep[1] is complete. Further molecular biological studies on the regulation of glycosyltransferases will be necessary to answer this question.

The current study also provided an additional insight on expression of epitopes defined by mAbs 1-1-M and 3F11. The two mAbs 3F11 and 1-1-M, respectively, have been known to bind to the Galβ1–4GlcNAc and GalNAcβ1–3Gal residue of the LOS that lacks elongation on the Hep[2] (10, 11). However, binding of the two mAbs to WG LOS (Fig. 6) showed that their epitopes are also expressed on LOS having a lactose attached to Hep[2]. These

**Table IV**

The results of mAb 2C7 binding to the LOSs whose Hep[2] is dibranched

| 2C7 binding | LOS | R₁ | R₂ |
|-------------|-----|----|----|
| +           | WG (minor) | GalNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1 | Galβ1–4Glcα1 |
| +           | WG (major) | Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1 | Galβ1–4Glcα1 |
| +           | 15253-Gal | Glcβ1 | Galβ1–4Glcα1 |
| −           | 15253 lgtE mutant | Glcβ1 | Galβ1–4Glcα1 |

\* JW31R LOS had been reported to contain Hex-Hex and HexNAc-Hex-Hex on Hep[1] and Hep[2], respectively (9), and the structures of R1 and R2 are proposed based upon the PAGE/blot results of enzyme-treated JW31R LOS samples.

\* The identities of R1 and R2 could be reversed.
results indicate that OS elongation patterns cannot be determined based solely on the binding of mAbs 1-1-M and 3F11. In summary, we determined that the major WG LOS has the following structure: a Galβ1–4GlcNAcβ1–3Galβ1–4Glc (lacto-neotetraose) and a Galβ1–4Glc (lactose), respectively, are linked to Hep[1] and Hep[2] of the GlcNAc-Hep[2]-Hep[1] trisaccharide core. mAb 2C7 binds to the inner 15253 LOS structure found within WG LOS. In addition, we found that JW31R LOS is also an elongated form of 15253 LOS containing extra disaccharide linked to the lactose on Hep[2] and that expression of the 2C7 epitope within JW31R LOS is blocked due to the substitution of the Hep[2]-lactose. Based on the structural data of these LOS and the results obtained from immunochemical analyses, we conclude the following: 1) mAb 2C7 requires both the 15253 OS as a minimum structure and the N-linked fatty acids in the lipoidal moiety for full expression of the epitope; 2) mAb 2C7 binds to LOS whose OS elongate from the lactose on Hep[1] of the 15253 OS but not on Hep[2]; and 3) the 2C7 epitope is expressed on gonococcal LOS despite the presence of human carbohydrate epitopes such as Hep[2]; and 3) the 2C7 epitope is expressed on gonococcal LOS in vivo (18), the epitope defined by mAb 2C7 could potentially be used as a safe site for the development of a vaccine candidate.

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