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Further Antigenic Similarities of *Neisseria gonorrhoeae* Lipooligosaccharides and Human Glycosphingolipids†

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Anticarbohydrate monoclonal antibodies were tested for their ability to bind to various strains of *Neisseria*. A monoclonal antibody that binds to the ganglio-series glycosphingolipid, ganglio-*N*-triaosylceramide, also bound to strains of *Neisseria gonorrhoeae* but not to other species of *Neisseria*. An antibody specific for the globo-series glycosphingolipid, globotriaosylceramide, also bound to strains of *N. gonorrhoeae*, *Neisseria meningitidis*, *Neisseria lactamica*, and *Branhamella catarrhalis* but not to any other strains of nonpathogenic *Neisseria*.

The lipooligosaccharides (LOS) of pathogenic and nonpathogenic *Neisseria* species are composed of multiple components that are different antigenically and chemically (5). A number of monoclonal antibodies (MAbs) that bind to epitopes in the LOS of *Neisseria gonorrhoeae* (17), *Neisseria meningitidis* (14, 17), *Neisseria lactamica* (13), and *Haemophilus influenzae* (21, 27) have been identified, and these epitopes are similar antigenically to epitopes on mammalian glycosphingolipids (GSL). One group of MAbs binds to lactoneo-series (Galβ1→4GlcNAcβ1→3Gal-R) GSL (18). The LOS epitope bound by these MAbs can be sialylated in gonococci (20), meningococci (19), and *H. influenzae* (21). A second group of MAbs presumably binds to terminal Galα1→4Gal residues in the LOS strains of *Haemophilus* and *Neisseria*. This conclusion was based on the specific inhibition of anti-LOS MAbs by Galα1→4Gal disaccharides (27) and the binding of a MAb specific for the GSL antigen, P^k (Galα1→4Galβ1→4Glc-ceramide), to strains of *H. influenzae* (21) and to a pyocin-resistant mutant strain of *N. gonorrhoeae* (8).

Recent chemical studies have confirmed that lacto-*N*-neotetraose (Galβ1→4GlcNAcβ1→3Galβ1→4Glc) (8, 29) and globotriaose (Galα1→4Galβ1→4Glc) (8) are part of the chemical structure of some gonococcal LOS components. These previous results suggested that there might be other examples of LOS antigenic mimicry. In an attempt to identify such antigens, a number of anticarbohydrate MAbs that bind to GSL have been analyzed for their ability to bind to strains of *Neisseria* and *Haemophilus*.

The MAbs were screened first in a solid-phase radioimmunoassay (17, 33) with whole bacteria as antigens. Strains of *Neisseria* were grown as described previously (23), and strains of *Haemophilus* were grown on plates of chocolate agar at 35°C (21). Bacteria were suspended in phosphate-buffered saline (PBS) to an optical density (at 620 nm) of 0.15. Polyvinyl microtiter wells were sensitized with the whole bacteria as described previously (1). Each anticarbohydrate MAb was tested at a range of concentrations with each bacteria. The MAbs tested were SH-34, 103HT30, Gal-13, 3D9 (anti-P^k), 7B6 (anti-P_1), 5C11 (anti-Gal-Gal), 2D4, and MC-480. MAbs 3D9, 7B6, and 5C11 were purchased from Accurate Chemical Inc. (Westbury, N.Y.). MAbs 2D4 and MC-480 were purchased from the American Type Culture Collection (Rockville, Md.) and the Developmental Studies Hybridoma Bank (Camden, N.J.), respectively.

The carbohydrate sequence specificities of the MAbs are shown in Table 1. Five of the eight MAbs bound to at least one of the strains or antigens. These MAbs were 2D4 (binds to asialo-GM2: GalNAcβ1→4Galβ1→4Glc-ceramide [cer]), anti-P^k (anti-Galα1→4Galβ1→4Glc-cer), anti-P_1 (anti-Galα1→4Galβ1→4GlcNAc-R), anti-Gal-Gal (anti-Galα1→4Gal-R), and SH-34 (binds to asialo-GM1: Galβ1→3GalNAcβ1→4Galβ1→4Glc-cer). The anti-P^k MAb had been shown previously to bind to the LOS of *H. influenzae* strains (21) and to an LOS of the gonococcal pyocin-resistant mutant strain 1291B (8). One of the two different anti-asialo-GM1 MAbs (SH-34 and 103HT30), MAb SH-34 bound to both gonococcal and meningococcal LOS, but 103HT30 did not bind to any strains tested. The terminal Galβ1→3GalNAc disaccharide presumably recognized by anti-asialo-GM1 was of interest, since this structure also can be O-linked to mucin glycoproteins (15) and is present in human cervical mucin (32). However, the results indicated that these two MAbs had very different specificities and suggested that MAb SH-34 was not specific only for ganglio-triaose. Also, MAb MC-480, specific for stage-specific embryonic antigen-3, which has a terminal Galβ1→3GalNAc disaccharide, did not bind to any of the strains. Because of its broad specificity, MAb SH-34 was not characterized further in this study. The variable amount of binding of some of the MAbs to different strains indicated that there was a wide range of epitope concentrations expressed among the strains (data not shown).

Table 2 shows the percentage of pathogenic and nonpathogenic *Neisseria* strains that bound MAb 2D4, anti-P^k, and anti-P_1. The anti-P^k MAb bound to strains of gonococci, meningococci, and *N. lactamica* and also to both strains of *Branhamella catarrhalis*. The largest percentage of neisserial strains binding the anti-P^k MAb (51%) were gonococci. The anti-Gal-Gal and anti-P_1 MAbs bound to only a few strains, and they bound to strains different from those that bound MAb anti-P^k, indicating that the bacterial epitopes binding the MAbs were different. The binding of anti-P_1

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TABLE 1. Binding of anticarbohydrate MAbs to strains of *Neisseria* in solid-phase radioimmunoassay

MAb	Structure recognized[a]	Common name[b]	Reference	Binding of MAb to strains of[c]:	
				Neisseria	*Haemophilus*
3D9	Galα1→4Galβ1→4Glc-cer	P^k	2, 21	+	+
7B6	Galα1→4Galβ1→4GlcNAc-R	P_1	2	+	−
5C11	Galα1→4Gal-R	Gal-Gal	22	+	NT
2D4	GalNAcβ1→4Galβ1→4Glc-cer	Asialo-GM2	31	+	−
SH-34	Galβ1→3GalNAcβ1→4Galβ1-4Glc-cer	Asialo-GM1	25	+	−
103HT30	Galβ1→3GalNAcβ1→4Galβ1-4Glc-cer	Asialo-GM1	7	−	NT
MC-480	Galβ1→3GalNAcβ1→3Galα1-R	SSEA-3	9	−	NT
Gal-13	Galα1→3Galβ1→4GlcNAcβ1-R	CPH	4	−	NT

[a] Only the terminal di- or trisaccharide of a GSL that bound the MAb is shown for MAbs 7B6, 5C11, MC-480, and Gal-13. Although this is a proposed specificity for the MAb, it is possible that the MAb binds to other similar structures with varying affinities.
[b] SSEA-3, stage-specific embryonic antigen-3; CPH, ceramide pentahexaoside (for CPH, R = 3Galβ1→4Glc-cer).
[c] Each MAb was tested with at least 20 strains, but not all of the same strains were tested with each MAb. +, at least one antigen bound the MAb; −, no antigens bound the MAb; NT, not tested.

suggested that a residue with galactose that was linked α to a Galβ1→4GlcNAcβ1→3Gal residue might be present in some gonococcal LOS. Structural analysis of a gonococcal LOS that is strongly positive for the anti-P_1 MAb will confirm whether this structure exists. MAb 2D4 (anti-asialo-GM2) bound to 54% of the gonococcal antigens tested. It did not bind to any strain of meningococci, *N. lactamica*, nonpathogenic *Neisseria*, or *H. influenzae*. These results indicated that the LOS epitope defined by MAb 2D4 was specific for strains of *N. gonorrhoeae*.

The results of previous studies characterizing other anti-GSL and anti-LOS MAbs (18, 27) implied that MAbs 2D4 and anti-P^k had probably bound to LOS. To confirm this, LOS were extracted from two strains that had bound the MAbs in solid-phase radioimmunoassay, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16), and then either stained with silver (26) or transferred to nitrocellulose for analysis with MAbs (17). After being incubated with the appropriate MAb, the nitrocellulose was washed and incubated with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin secondary antibody (24).

Figure 1 shows the silver stain and immunoblot of the purified LOS from *N. gonorrhoeae* GC33 and *N. meningitidis* group Y 8032. The LOS of the gonococcal strain GC33 is heterogeneous and, therefore, migrated diffusely in SDS-PAGE (Fig. 1A, lane 1). The anti-P^k MAb bound two closely migrating bands within the diffuse mixture of LOS (Fig. 1B, lane 1); these bands represented the more slowly migrating molecules shown in the diffuse silver-stained LOS (Fig. 1A, lane 1). Anti-P^k did not bind to 8032 LOS (Fig. 1B, lane 2), which was included as a molecular weight marker and as a negative control. MAb 3F11, which binds to a 4.5-kDa LOS band present in most gonococcal and group B and C meningococcal strains (17), bound to a component of GC33 LOS that migrated slightly ahead of the bands that bound anti-P^k (data not shown).

The binding of anti-asialo-GM2 (2D4) to the LOS of gonococcal strains 15373 and F62 is shown in Fig. 2. The LOS components of strains 15373 and F62 that bound anti-asialo-GM2 (Fig. 2A, lanes 2 and 3) had molecular masses of approximately 5.2 and 5.1 kDa, respectively. The MAb did not bind to meningococcal strain 8032 LOS (negative control). Anti-asialo-GM2 also bound to strain F62 LOS that migrated diffusely above the major 5.1-kDa band (Fig. 2B, lane 3).

Finally, since the anti-P^k and anti-asialo-GM2 MAbs were tested as ascites fluids, it was important to confirm that the antibody binding specifically to LOS was the same as the antibody binding to the carbohydrate of the GSL. For this analysis, purified asialo-GM2 was preincubated with anti-asialo-GM2 MAb and the inhibition of the binding of the antibody to purified F62 LOS was determined. Asialo-GM2 was diluted in a solution of chloroform-methanol-hexane (1:25:5.25) containing 0.08% polyisobutylmethacrylate (Polysciences Inc., Warrington, Pa.), and 10-μl samples

TABLE 2. Activity of the anti-P^k, anti-P_1, and anti-asialo-GM2 MAbs with strains of various species of *Neisseria* and of *B. catarrhalis* in solid-phase radioimmunoassay

Species	Activity with[a]:								
	Anti-P^k			Anti-P_1			Anti-asialo-GM2		
	Total no. of strains	No. positive	% Positive	Total no. of strains	No. positive	% Positive	Total no. of strains	No. positive	% Positive
N. gonorrhoeae	70	36	51	70	8	11	44	24	54
N. meningitidis	63	6	9	12	0	0	12	0	0
N. lactamica	10	3	30	10	0	0	10	0	0
Other *Neisseria* spp.[b]	10	0	0	NT	NT	NT	10	0	0
B. catarrhalis	2	2	100	NT	NT	NT	2	0	0

[a] A strain was considered positive if it bound >10% the amount of MAb that resulted in maximum binding to a positive control antigen. NT, not tested.
[b] Other nonpathogenic *Neisseria* spp. tested were *Neisseria sicca* (two strains), *Neisseria cinerea* (two strains), *Neisseria mucosa* (two strains), and *Neisseria perflava* (four strains).

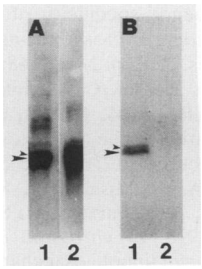

FIG. 1. Binding of anti-P^k MAb to LOS. LOS of *N. gonorrhoeae* GC33 (lane 1) and *N. meningitidis* 8032 (lane 2) were separated by SDS-PAGE, and then the LOS were silver stained (A) or transferred to nitrocellulose and immunostained with anti-P^k (B). The arrowheads point to the closely migrating major and minor bands positive for anti-P^k. Anti-P^k does not bind to strain 8032 LOS (negative control).

containing 40 to 2,500 ng of GSL were added to microtiter wells. After the solvent evaporated, the wells were washed with a blocking reagent (1% casein–10 mM Tris–150 mM NaCl–30 mM sodium azide, pH 7.5), MAb 2D4 (anti-asialo-GM2) was added to each well (approximately 20 ng), and the wells were incubated for 14 h at room temperature. The liquid was removed from each well and transferred to a second well sensitized with purified F62 LOS (50 μg/ml), and the wells were incubated for 2 h at room temperature. The liquid was removed, each well was washed with blocker and PBS (50 mM, pH 7.2), and then the amount of MAb bound was detected as described previously (17). In this assay, 50% of anti-asialo-GM2 was inhibited from binding to purified F62 LOS by approximately 200 ng of purified asialo-GM2 (data not shown). There was no inhibition with asialo-GM1 in a similar assay. MAb 2D4 bound only to a single band of the purified, and thin-layer-chromatography-separated, asialo-GM2; it did not bind to asialo-GM1 (data not shown) (3). In a similar assay, the anti-P^k MAb could be prevented from binding to purified GC33 LOS by Galα1→4Gal disaccharide linked to bovine serum albumin (BSA) by a cross-linker (Carbohydrates International, Arlöv, Sweden). The Galα1→4Gal-BSA at a concentration of 50 μg/ml inhibited 50% of the maximal binding of approximately 2 μg of MAb anti-P^k per ml to a microtiter well sensitized with LOS (50 μg/ml). These results confirmed that the antibodies binding to the LOS were the same as those specific for the carbohydrate epitopes of the GSL.

The anti-asialo-GM2 MAb bound only to gonococci (Table 2) and only to strains possessing an LOS component with a molecular mass of ≥5.1 kDa. This LOS component is identical to (or comigrates in the same region as) the LOS that binds MAb 1-1-M (17, 30). Both MAbs bind only to LOS of gonococci, indicating that they recognize strain-specific epitopes; however, their binding patterns with different gonococcal strains indicate that the MAbs bind to separate epitopes (data not shown). In contrast, the anti-P^k MAb bound to both pathogenic and nonpathogenic strains of *Neisseria* (Table 2) and, as shown in previous studies, to strains of *H. influenzae* (21). However, a structure apparently similar to P^k and present on *H. influenzae* LOS (digalactoside; 4C4 epitope) (27) has been implicated in virulence. These results suggest either that the epitopes are different or that the epitopes play a different role on different species of mucosal pathogens. Possibly, the P^k-like antigen on *Neisseria* strains is related to the organism's ability to exist as a commensal and/or in a state of temporarily asymptomatic infection. The P^k antigen is present in a number of human tissues, including cells of the uroepithelial system (12).

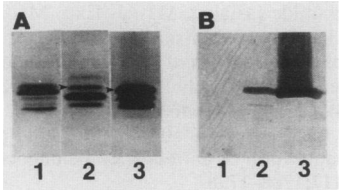

FIG. 2. Binding of anti-asialo-GM2 MAb (2D4) to LOS. LOS of *N. meningitidis* 8032 (lane 1) and *N. gonorrhoeae* 15373 (lane 2) and F62 (lane 3) were separated in SDS-PAGE and silver stained (A) or transferred to nitrocellulose and immunostained with anti-asialo-GM2 (B). The arrowheads point to the bands that bind anti-asialo-GM2. Note the diffusely migrating LOS in lane 3 (A) that binds MAb 2D4 (B).

The binding of these anticarbohydrate MAbs to LOS indicates only that the bacterial LOS are similar antigenically to human GSL. However, recent studies have shown conclusively that a pentaose in the larger component of strain F62 LOS is as follows: GalNAcβ1→3Galβ1→4Glc NAcβ1→3Galβ1→4Glc, which is V^3-(β-*N*-acetylgalactosaminyl)lactoneotetraose (29), a structure which is identical to pentaoses of the X_2 (10) and asialo-G_3 (28) GSL present in human erythrocyte membranes. This would be consistent with the results of subsequent studies in which MAb 2D4 bound to the X_2 GSL as well as to asialo-GM2 (11). Thus, it is probable that MAb 2D4 bound to the GalNAcβ1→3Gal residue at the terminus of the large mass component of F62 LOS.

Galactose that is linked α1→4 to galactose of lactosylceramide (Galβ1→4Glc-cer) defines the globo-series GSL present in mammalian cells; Galβ1→4GlcNAc that is linked β1→3 to galactose of lactosylceramide defines the neolacto-series GSL (6). The presence of multiple LOS structures that mimic human GSL would provide the gonococcus with molecular diversity within the varied environments encountered in humans. MAb-defined LOS differences among strains of *Neisseria* and *Haemophilus* that can be related to differences in the glycoconjugate network on the surface of cells in these environments might be helpful in achieving a better understanding of the mechanisms of pathogenesis of these bacteria.

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