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Epitope Specificities of the Group Y and W-135 Polysaccharides of *Neisseria meningitidis*▽

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Previous studies have identified the length dependency of several polysaccharide (PS) protective epitopes. We have investigated whether meningococcal polysaccharides Y and W-135 possess such epitopes. Oligosaccharides (OSs) consisting of one or more disaccharide repeating units (RU) were derived from the capsular PSs of group Y and W-135 meningococci (GYMP and GWMP, respectively) by mild acid hydrolysis. The relative affinities of anticapsular antibodies binding to derivative OSs of different chain lengths were measured in inhibition enzyme-linked immunosorbent assays. As OS size increased from two to three RU, there was a notable increase in binding inhibition of rabbit anti-group Y antiserum. This pattern of antibody binding inhibition was also observed for rabbit antiserum to group W-135, though the inhibition increase was much more pronounced. In the cases of both OS species, the concentration of inhibiting antigen required to achieve 50% inhibition of rabbit immunoglobulin binding increased progressively as the inhibiting disaccharide chain length increased from 1 RU through greater than 50 RU. These data suggest that antibodies directed against both of these meningococcal PSs recognize conformational epitopes only fully expressed in higher-molecular-weight forms of these antigens.

Meningococcal disease, caused by *Neisseria meningitidis*, occurs mainly as either septicemia or meningitis, and is a worldwide health problem (23). The main virulence factor of *N. meningitidis* is its capsular polysaccharide (CPS), which protects it against complement-mediated bactericidal activity and opsonization (11, 17). Immunity to infection with many encapsulated bacteria is conferred by antibodies to the CPS (7, 18, 26).

N. meningitidis strains have been classified into at least 13 serogroups on the basis of the immune specificity of the PS capsule (25). Invasive infections are most frequently caused by five of these serogroups—A, B, C, Y, and W-135 (2). The proportion of meningococcal cases in the United States caused by serogroup Y has increased dramatically from 2% during 1989 to 1991 to 37% during 1997 to 2002 (2). Serogroup W-135, spread in association with the Hajj pilgrimage, has caused outbreaks and isolated cases in many countries, including the United States (4, 21). Of all cases of meningococcal disease in the United States among persons older than 11 years of age, 75% are caused by serogroup C, Y, or W-135 (2).

The CPSs are made up of either monosaccharides making a homopolymer, or from repeating units (RU) normally consisting of two to six sugar residues (30). CPSs vary significantly in their ability to stimulate specific antibody. As a very general rule, PSs with a molecular mass of >90,000 kDa are good immunogens in adults, while those with a molecular mass of <50,000 kDa are poorly immunogenic (8, 15, 20, 28). Coupling a PS or component oligosaccharide (OS) to a carrier protein to produce a conjugate molecule may result in immunogenic properties more like those of thymus-dependent antigens, including stimulation of higher levels of immunoglobulin G antibodies, enhanced memory responses, and immunogenicity in infants.

The biochemical basis of immunogenicity to bacterial CPS has been extensively studied. Immunity following meningococcal infection is serogroup specific (33). Susceptibility to systemic disease is linked to an absence of detectable bactericidal antibodies (7). Antibody responses can be greatly affected by their physicochemical properties: e.g., molecular size, specific determinants, and conformation (19). Both the group Y meningococcal polysaccharide (GYMP) and group W-135 meningococcal polysaccharide (GWMP) are highly immunogenic and are currently used as components of a vaccine against meningococcal meningitis in humans (10, 16). In addition, a conjugate vaccine containing these serogroups is now available (2).

Antibacterial PS-specific antibodies are generally of low intrinsic affinity (9). Enhancement of antibody binding can be observed if the target antigen has a repetitive structure, making those polymers functionally multivalent (9). Evidence for length-dependent conformational epitopes had previously been reported for group B streptococcus type III (3, 31, 34), group B *N. meningitidis* (13), *Streptococcus pneumoniae* type 14 (32), and *Haemophilus influenzae* type b (24) polysaccharides. It had been considered, in fact, that the recognition of conformational epitopes may be a more general phenomenon than previously appreciated in the interaction of anti-PS antibodies with bacterial CPS antigens (32). It has been speculated that

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more stable conformational epitopes of PS species can result from the restriction of rotational movement of individual sugar residues and that these conformations may be preferred by antibodies (24).

In this study, we examined the nature of the group-specific epitopes present on GYMP and GWMP. Both of these PSs contain sialic acid. Interaction of sialic acid with the PS backbone had previously been shown to be important in defining the conformational epitope of group B *Streptococcus* polysaccharide III (3).

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MATERIALS AND METHODS

Growth of group Y and W-135 meningococcal strains. Meningococcal Slaterus Y strain and meningococcal W-135 S4383 strain were provided by Carl Frasch (CBER/FDA, Bethesda, MD). The strains were grown in shake flasks under agitation at 37°C in a modified Franz medium containing glucose and yeast extract. Cultures were harvested by centrifugation at 8,000 rpm, and supernatants were collected and filtered through 0.22-μm-pore filter units. The native Slaterus Y PS is 93% O acetylated (6). The W-135 strain expressed PS lacking O-acetyl groups as shown by ^{1}H nuclear magnetic resonance (^{1}H-NMR) on the purified PS.

Preparation of GYMPs and GWMPs. Microfiltered culture supernatants were concentrated by ultrafiltration using a filter device with a Biomax 300-kDa Pellicon membrane (0.5 m^2) (Millipore Corp., Bedford, MA). The concentrated retentate was diafiltered 12 times against 1 M NaCl and then 10 times against deionized water and freeze-dried. The GYMP was then treated with mild base (0.1 N NaOH) at room temperature overnight and further purified by ultrafiltration to yield the de-O-acetylated high-molecular-weight form of the PS.

The high-molecular-weight purified "native" PSs were analyzed by gas chromatography-mass spectrometry with an Agilent 6890 gas chromatograph/5973 mass selective detector with a 30-m HP capillary column for sugar composition and by ^{1}H-NMR spectroscopy at 500 MHz using a Bruker AMX-500 spectrometer. The sizes and polydispersity of the PS were determined using size exclusion chromatography (SEC) on line with the multiangle laser light-scattering (MALLS) detector (miniDawn; Wyatt Technology) (5).

Molecular modeling of group Y and W polysaccharides. Molecular modeling was carried out using the GEGOP (GEometry of GlycOProteins) program. GEGOP utilizes the modified HSEA (Hard Sphere Exo-Anomeric) force field in combination with Metropolis Monte Carlo calculations (27). Typically, the global minimum of each individual glycosidic linkage was calculated and the PS was constructed using the dihedral angles that correspond to the global minimum. If the PS chain could not be extended due to collision between the head and tail of the chain, the next local minimum was used to construct the PS chain. The PS (up to 10 RU) was finally minimized.

Preparation of PS-HSA conjugates. The human serum albumin (HSA; Sigma, St. Louis, MO) conjugates used for enzyme-linked immunosorbant assays (ELISAs) were prepared by reductive amination using either GYMP or GWMP. Each oxidized fragment of ~10 kDa was added to HSA followed by reduction with $NaBH_3CN$ as previously described (12).

Generation of OS inhibitors of discrete lengths. The GYMP was partially hydrolyzed with 0.1 N sodium acetate buffer (pH 3.0) at 80°C over a 2-h time frame. The OSs produced by mild acid hydrolysis have sialic acid as a terminal reducing sugar. The generated OSs of discrete length were then separated by SEC on a Superdex G-30 column (Pharmacia). The elution of the oligosaccharides was monitored by UV detection at 214 nm. Fractions containing each OS of a discrete length were combined and lyophilized. The purity and size of each of the OS inhibitors were determined by ^{1}H-NMR spectroscopy at 500 MHz on a Bruker Instruments AMX 500 spectrometer (Billerica, MA). The same protocol was followed to generate GWMP OSs of discrete lengths. The ^{1}H-NMR data were used to prove the presence of NeuAc at the reducing end of both the GYMP and GWMP OSs, as well as measuring their degree of polymerization (1).

Antisera. Reference typing rabbit antisera specific for GYMP, GWMP, group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) were obtained from DIFCO (Becton Dickinson, Franklin Lakes, NJ).

ELISAs for binding of antibody and inhibition of antibody binding. ELISAs for binding and inhibition of binding of rabbit antisera to microtiter plates coated with either GYMP-HSA or GWMP-HSA conjugates were performed as described previously (22).

RESULTS

Structures of GYMPs and GWMPs. The repeating disaccharide subunit structures of the GYMPs and GWMPs are shown in Fig. 1a. The GYMP is a repeating →6)-α-D-Glc(1→4)-α-D-NeuAc(2→ disaccharide, while the GWMP is a repeating →6)-α-D-Gal(1→4)-α-D-NeuAc(2→ disaccharide. A cursory examination of the nearly identical primary sequence and linkage positions of the GYMP and GWMP suggests that the two PSs will adopt nearly identical three-dimensional topologies, with the exception of the surface about carbon 4 of the hexose sugar, where the only structural difference exists between the two polymers. In the Y PS, the hydroxyl group at C4 is oriented in an equatorial position ("-eq" in H3eq/ax), while in W-135 antigen, this hydroxyl group is axial ("-ax" in "H3eq/ax"). It would be anticipated that surfaces composed of the sialic acid residue and parts of the hexose residue would be nearly identical. Modeling supports these expectations.

GYMP has a helical structure in the lowest energy conformation with four residues making one full turn. This arrangement creates identical surfaces on opposite sides of the molecule. Alternating RU make up these opposite surfaces. Figure 1b shows a space-filling model of the group Y PS structure. There is a hydrophobic surface (green arrow) on the lower left side and the upper right side (cannot be seen in this view) of the model. On the top and the bottom sides is the hydrophilic surface (blue arrows). For the group Y antigen, the O-4 atom of Glc disrupts the hydrophobic surface that is seen in group W-135 antigen (Fig. 1c). This is the largest structural difference between the two PSs that occurs on exposed surfaces, but the hydrophilic grooves are also different. GWMP has a related helical structure in the lowest energy conformation, with four residues making one full turn. Alternating RU make up these opposite surfaces. Figure 1c displays a model of the structure of the GWMP. Like the GYMP, there is a hydrophobic surface (green arrow) on the left side and the right side (which cannot be seen in this figure) of the model. On the top and the bottom sides is the hydrophilic surface (shown by red arrows).

In both Fig. 1b and c, the similarities of surfaces that involve the NeuAc acetamido and the exocylic carbon chain C7-C9 are nearly identical in their topologies. In the absence of immunological properties that might direct the immune response away from recognition of the sialic acid residues in each polysaccharide, it might be anticipated that sera induced by the two antigens would include populations of antibody that are highly cross-reactive and another population directed toward the surface containing C-4 of the hexose residue that would be highly specific. However, in reality in the case of the cross-reacting specificities, this is not significant, as serogrouping antisera produced in rabbits are typically used without cross-adsorption with related serogroups of meningococci (29).

Specificity of rabbit antisera on GYMP-HSA- and GWMP-HSA-coated plates. The specificity of rabbit antisera to the coated conjugate antigens was examined. Because the structures of GYMP and GWMP are so similar, antibodies pro-

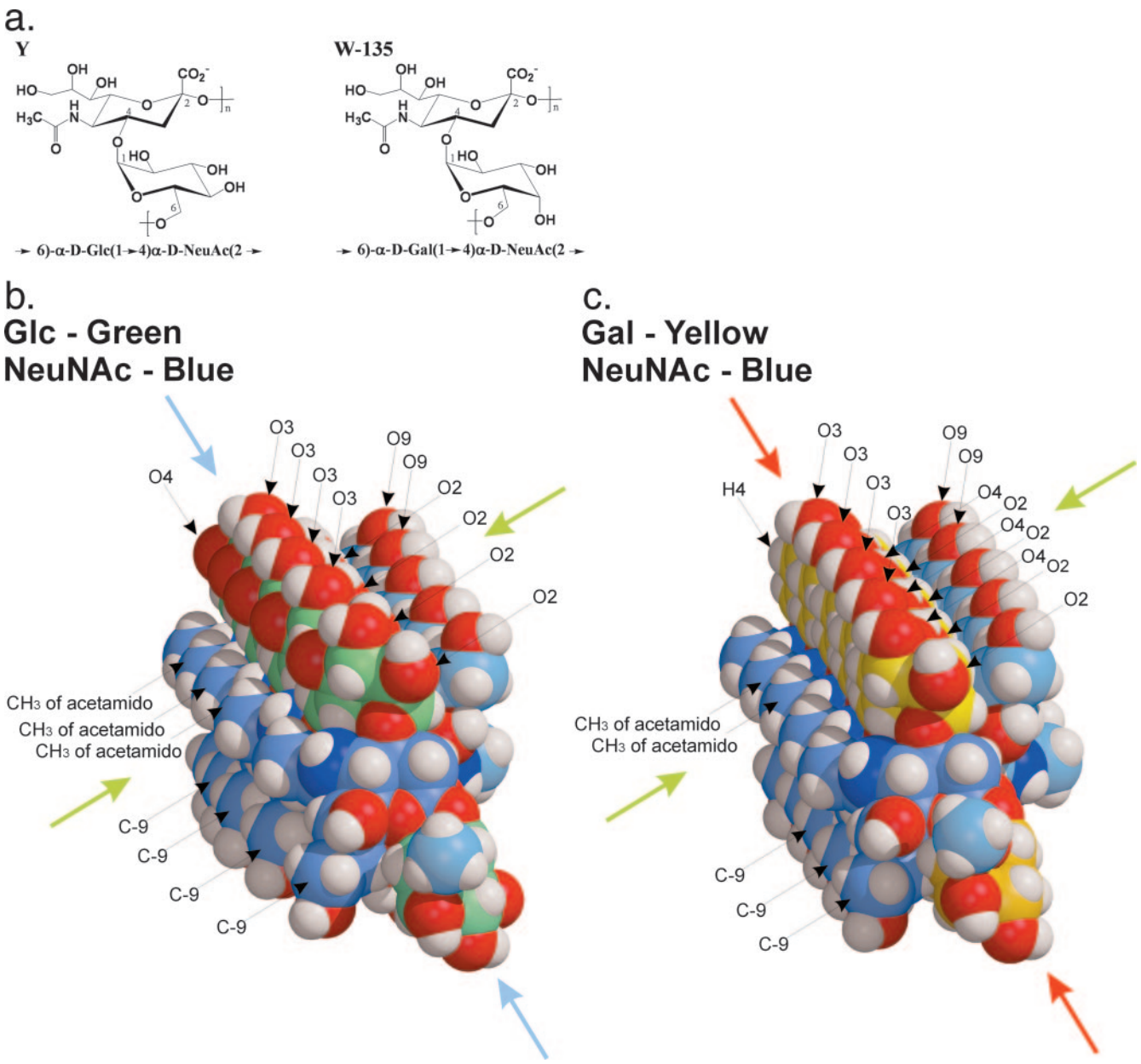

FIG. 1. (a) Structures of meningococcal polysaccharides. (b) Space-filling model of *Neisseria meningitidis* group Y polysaccharide structure. Green arrows indicate hydrophobic surfaces; blue arrows indicate hydrophilic surfaces. (c) Space-filling model of *Neisseria meningitidis* group W-135 polysaccharide structure. Green arrows indicate hydrophobic surfaces; red arrows indicate hydrophilic surfaces.

duced against one of these PSs might be expected to cross-react with the other. Rabbit antisera to groups B, C, Y, and W-135 were serially diluted on a microtiter plate coated with GYMP-HSA conjugate (Fig. 2a). The ELISA titration curves display the strong reactivity of the anti-group Y antiserum and no corresponding cross-reactivity to the coated solid phase with the anti-group B, C, and W-135 antisera. Similarly, the same four rabbit antisera were serially diluted on a microtiter plate coated with GWMP-HSA (Fig. 2b). There is strong reactivity displayed by the anti-group W-135 antiserum and no significant cross-reactivity from any of the other three serotyping antisera.

Characterization of the de-O-acetylated GYMP and GWMP OS inhibitors. The purity and size of each of the OS inhibitors were determined by high-resolution ^{1}H-NMR spectroscopy and SEC.

The proton chemical shift (δ) assignments for some of the signals of the GYMP and GWMP OS repeating units are collected in Table 1 and Table 2, respectively. The resonances assigned correspond to anomeric protons (H1) of the Gal/Glc residues, the H3eq/ax, and the N-acetyl protons ($NHCOCH_3$) of NeuAc residues of each of the RU shown in Fig. 1a. The assignments were made by comparison with published data (1, 14), and some of them were confirmed by use of two-dimen-

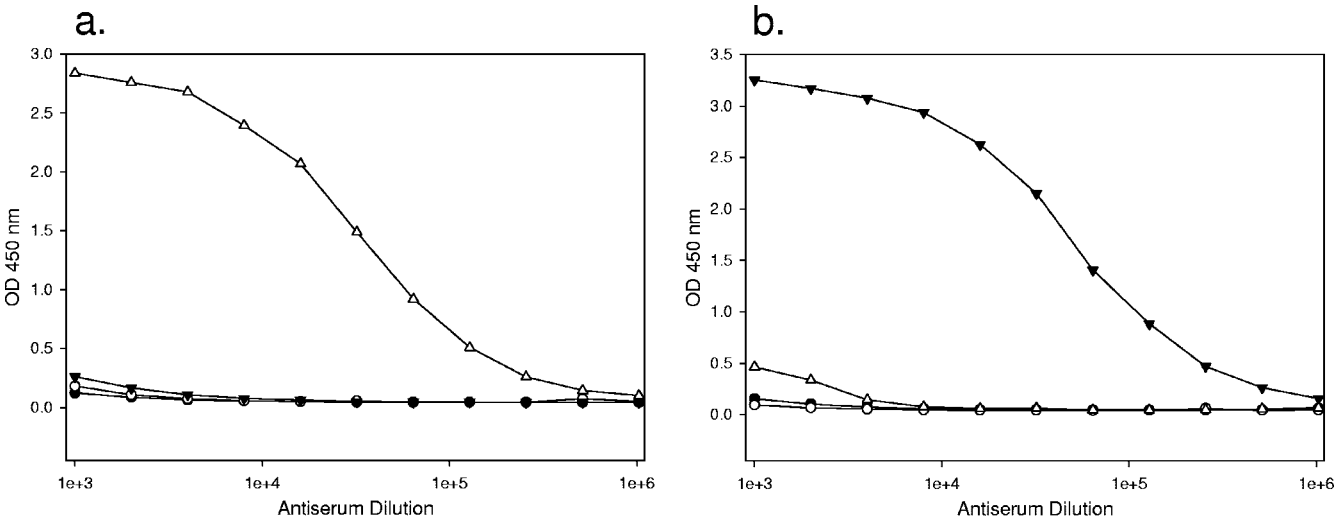

FIG. 2. (a) ELISA titration of rabbit antisera on GYMP-HSA-coated plate. (b) ELISA titration of rabbit antisera on GWMP-HSA-coated plate. ●, anti-*N. meningitidis* group B; ○, anti-*N. meningitidis* group C; ▼, anti-*N. meningitidis* group W-135; △, anti-*N. meningitidis* group Y. OD 450, optical density at 450 nm.

sional proton-proton (correlation spectroscopy [COSY]) and proton-carbon (heteronuclear single-quantum correlation [HSQC]) correlation experiments (data not shown). These NMR data prove the presence of NeuAc at the reducing end of both the GYMP and GWMP oligosaccharides, thus validating the basis for the degree of polymerization estimation.

Determinant specificity of the GYMP. Inhibition of binding of rabbit anti-group Y antiserum to GYMP-HSA-coated plates using OSs ranging from 1 to approximately 55 disaccharide RU of GYMP was examined (Fig. 3). High-molecular-weight GWMP, used as a related PS inhibitor, did not inhibit the binding of the rabbit anti-group Y antiserum. One group Y disaccharide RU was a very poor inhibitor of antibody binding to the solid phase, while the binding inhibition increased markedly with 2 RU. It is unclear whether 100% inhibition of antibody binding could be achieved using 2 RU. There was complete inhibition of antibody binding when the subunit size was 3 RU or greater. Progressively increasing the size of the oligosaccharide from 3 RU through an estimated 55 RU progressively increases the relative affinity of the inhibition curves.

Determinant specificity of the GWMP. Binding inhibition of rabbit anti-group W-135 antiserum to GWMP-HSA-coated microtiter plates using OSs ranging from 1 RU to an estimated 66 RU of GWMP was also examined (Fig. 4). As with the group Y oligosaccharide subunits, inhibitors of 1 and 2 RU of GWMP were very poor inhibitors. In this experiment, there was a more dramatic increase in binding inhibition going from the 2-RU inhibitor to the 3-RU inhibitor than there was in the similar inhibition study conducted with anti-group Y antiserum (Fig. 3). Increasing the size of the GWMP subunit inhibitors

TABLE 1. Proton chemical shift in NMR analysis for GYMP oligosaccharides

Saccharide type	Signal pattern[b] (no. of RU)	Chemical shift[a] (RU element)			
		Glc H1	NeuAc		
			H3a	H3e	CH_3(NAc)
OS	ab (1)	5.055 (a)	1.90 (b)	2.35 (b)	2.04 (b)
	a′b′ab (2)	5.055 (a) 5.00 (a′)	1.78 (b) 1.68 (b′)	2.37 (b) 2.90 (b′)	2.05 (b) 2.02 (b′)
	a″b″a′b′ab (3)	5.06 (a) 5.025 (a′) 5.00 (a″)	1.78 (b) 1.68 (b′) 1.68 (b″)	2.36 (b) 2.89 (b′) 2.89 (b″)	2.055 (b) 2.035 (b′) 2.02 (b″)
	a‴b‴a″b″a′b′ab (4)	5.06 (a) 5.025 (a′) 5.025 (a″) 5.00 (a‴)	1.78 (b) 1.685 (b′) 1.685 (b″) 1.685 (b‴)	2.375 (b) 2.885 (b′) 2.885 (b″) 2.885 (b‴)	2.055 (b) 2.032 (b′) 2.032 (b″) 2.02 (b‴)
PS	ab	5.02 (a)	1.67 (b)	2.87 (b)	2.03 (b)

[a] Values are parts per million and correspond to anomeric protons (H1) of the Glc residue, the H3eq/ax, and the N-acetyl protons of the NeuAc residues.
[b] a, hexose; b, NeuAc.

TABLE 2. Proton chemical shift in NMR analysis for GWMP oligosaccharides

Saccharide type	Signal pattern[b] (no. of RU)	Chemical shift[a] (RU element)			
		Gal H1	NeuAc		
			H3a	H3e	CH_3(NAc)
OS	ab (1)	5.08 (a)	1.79 (b)	2.40 (b)	2.07 (b)
	a′b′ab (2)	5.08 (a) 5.10 (a′)	1.79 (b) 1.68 (b′)	2.405 (b) 2.93 (b′)	2.06 (b) 2.11 (b′)
	a″b″a′b′ab (3)	5.08 (a) 5.065 (a′) 5.10 (a″)	1.78 (b) 1.67 (b′) 1.67 (b″)	2.41 (b) 2.89 (b′) 2.93 (b″)	2.04 (b) 2.09 (b′) 2.11 (b″)
	a‴b‴a″b″a′b′ab (4)	5.08 (a) 5.08 (a′) 5.08 (a″) 5.10 (a‴)	1.79 (b) 1.68 (b′) 1.68 (b″) 1.68 (b‴)	2.41 (b) 2.89 (b′) 2.89 (b″) 2.94 (b‴)	2.05 (b) 2.10 (b′) 2.10 (b″) 2.11 (b‴)
PS	ab	5.06 (a)	1.68 (b)	2.89 (b)	2.09 (b)

[a] Values are in parts per million and correspond to anomeric protons (H1) of the Gal residue, the H3eq/ax, and the N-acetyl protons of the NeuAc residues.
[b] a, hexose; b, NeuAc.

progressively increased the apparent affinity of the inhibition curves.

DISCUSSION

We hypothesize that the rudimentary antibody binding sites for the GYMP and GWMP consist of 5 to 6 sugar residues, or 2.5 to 3 RU. The sixth residue, the reducing sialic acid, would likely be in equilibrium between the alpha and beta conformations depending whether it was unbound or bound by antibody, respectively. The fact that small OSs (3 RU) completely inhibited antibody binding to the both GYMP and GWMP at high OS concentrations indicates that the same antibodies bound to the smaller fragments as the larger PS but bound with lower apparent affinities. These results suggest that antibodies to both GYMP and GWMP bound to epitopes that were stabilized by higher-molecular-weight forms of the PS. The conformations of those epitopes in the lower-molecular-weight forms of these PS were less favorable for antibody binding, resulting in the lower-affinity interactions. In addition, the density of the stabilized epitope would likely increase with increasing chain length.

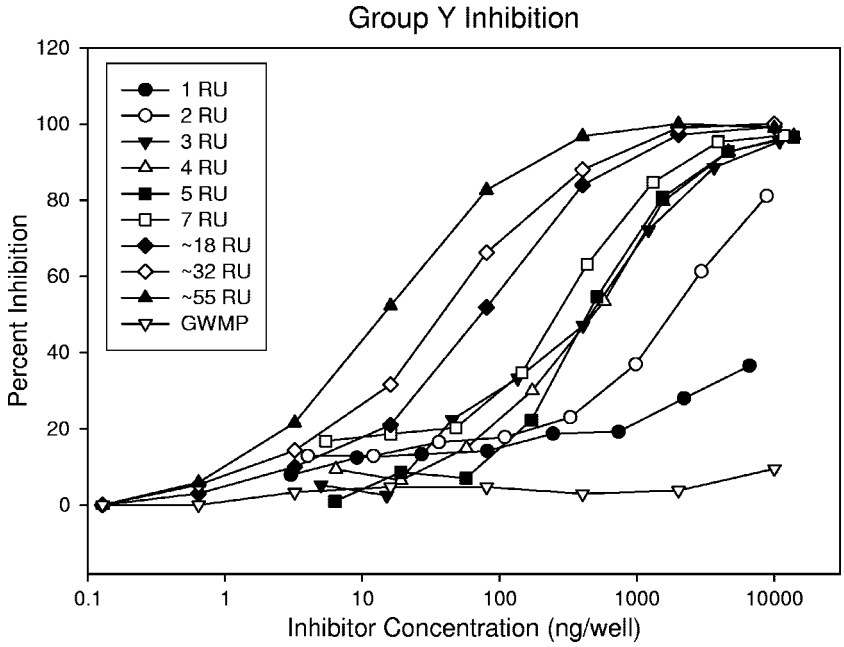

FIG. 3. Inhibition ELISA of rabbit anti-N. meningitidis Y antiserum on GYMP-HSA-coated plate. The mean molecular weight of larger fragments (≥18 RU) was determined by SEC-MALLS.

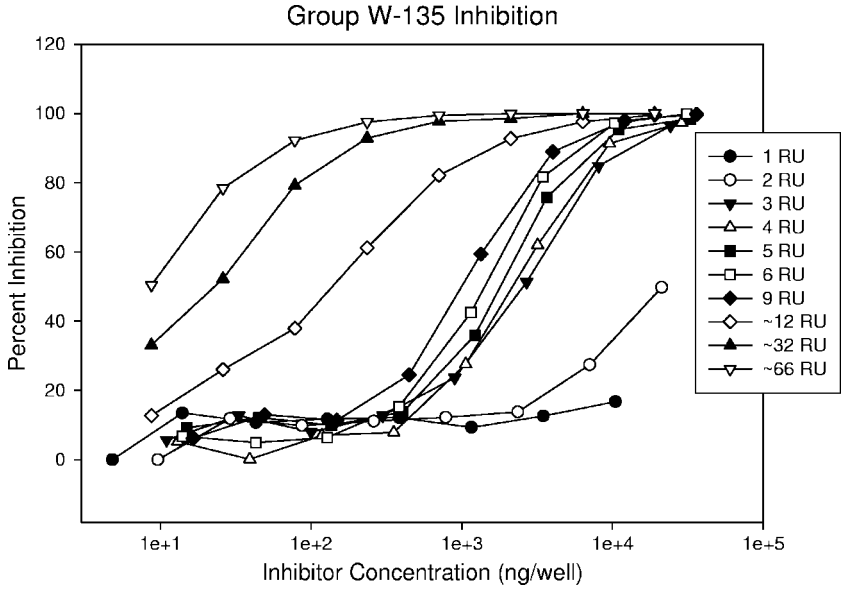

FIG. 4. Inhibition ELISA of rabbit anti-N. meningitidis W-135 antiserum on GWMP-HSA-coated plate. The mean molecular weight of larger fragments (≥12 RU) was determined by SEC-MALLS.

It is possible that the bivalent nature of the antibody molecules could lead to an increase in apparent avidity through monogamous bivalency. This effect would likely be maximized at an antigen chain length that would accommodate bivalent antibody binding to the same OS chain—roughly 10 to 20 sugar residues, or 5 to 10 RU (31)—and would not be a factor in apparent avidity increases beyond that OS chain size. Our results, however, clearly demonstrated an increase in apparent antibody avidity beyond those minimal OS chain sizes, thereby lending credence to a conformational epitope model for both the GYMP and GWMP antigens.

Another possibility is that bivalent antibody molecules could be capable of cross-linking epitopes on different OS chains in the same reaction mixture, thereby leading to a further increase in the apparent avidity of the antibody-OS interactions. In this case, the total antibody inhibition would continue to increase in ever smaller increments (as observed) with increasing OS chain length, approaching a theoretical maximum that could only be achieved at complete bivalent antibody binding.

The increase in apparent avidity could also be due to clumping of antibodies, as a result of noncovalent association, which may or may not be physiologically significant. In addition, antibodies with binding site specificity for a small OS may functionally discriminate between simple haptens consisting of those OSs and a PS of multiple RU of the same OS (29). High-affinity immune recognition of the bacterial CPS, rather than smaller OS fragments, would lead to activation of immune effector mechanisms (31).

NMR analyses of the inhibitors indicated minor conformational changes up through 4 RU for both OS species. What were not seen by NMR were the contributions that bound antibody molecules might make to the larger OS fragments and the stability that bound antibody might lend to the tertiary PS structures.

In summary, the conformational epitope model was more apparent for GWMP than GYMP since there was over a 300-fold increase in antibody inhibition, as the size of GWMP inhibitor grew from 3 RU to approximately 66 RU, compared to only a 35-fold increase, from 3 RU to approximately 55 RU, in the GYMP ELISA inhibition. The mechanism for this increase in inhibition, however, is not entirely clear. It would appear that the antibody binding to an antigenic site on a PS facilitated subsequent binding of other immunoglobulin molecules to the same PS, suggesting that these antibodies recognized conformational epitopes that are only fully expressed in high-molecular-weight forms of both PSs, although bivalent interactions may provide a contribution at higher PS concentrations.

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