Physicochemical Properties of *Neisseria meningitidis* Group C and Y Polysaccharide Antigens

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Chemical and physical studies of the *Neisseria meningitidis* group C and Y antigens indicated that these preparations are acidic polysaccharides. The group C antigen contains predominately N-acetyl neuraminic acid, with small amounts of glucose, glucosamine, and mannose, whereas the group Y antigen contains glucose, glucosamine, and mannose. The molecular weights of the group C and Y antigens were found to be 88,000 and 197,000, respectively. The group C antigen was susceptible to neuraminidase digestion. Finally, the immunological reactivity of neither antigen was inhibited in hemagglutination or immunodiffusion systems by neutral or substituted sugars.

Recent interest in the development of meningococcal polysaccharide antigens for use as human immunogens necessitates detailed studies of their immunological, biological, chemical, and physical properties (3). The group C and Y organisms are presently of clinical interest because of the predominance of the former in clinical disease and the latter in carrier rate studies (17, 18, 27). The isolation and immunochemical properties of the group C and Y polysaccharide antigens have been previously described (20). We have completed chemical and physical studies of these antigens by colorimetric analysis, gas chromatography, infrared spectroscopy, electrophoresis, sedimentation, velocity, and sedimentation equilibrium. The results indicate that these antigen preparations are heterosaccharide polymers with distinct physical and chemical differences.

**MATERIALS AND METHODS**

**Antigens.** Clinical isolates of *N. meningitidis* group C and primary nasopharyngeal isolations of group Y organisms were used for antigen preparation by the method of Robinson and Apicella (20).

**Antisera.** Rabbit antisera against *N. meningitidis* groups C and Y were obtained from the National Communicable Disease Center (NCDC), Atlanta, Ga., or by immunization of New Zealand albino rabbits as described previously (20).

**Chemical analysis.** Antigen samples for analysis were dialyzed against distilled water, lyophilized, and dried over P₂O₅. Total nitrogen determinations were done on a F and M carbon-hydrogen-nitrogen analyzer with glucosamine hydrochloride as reference standard. Colorimetric assays for pentoses were done by the method of Dische (7), 6-deoxyhexoses by the cysteine-sulfuric acid reaction (9), hexoses by the anthrone method (23), and heptoses by the method of Dische (8). Hexosamines were estimated by the method of Rondle and Morgan (21), with glucosamine hydrochloride treated under similar conditions as standard. N-acetyl hexosamines were estimated by the methods of both Aminoff, Morgan, and Watkins (1) and Reissig, Strominger, and LeLoir (19), with N-acetyl glucosamine (Sigma Chemical Co.) as standard. Total uronic acids were determined by the carbazole method (6), with a D-glucuronic acid as standard. Total sialic acids were assayed by the modified resorcinol technique (4). 2-Keto-3-deoxy-sugars were determined by the thiobarbituric acid assay of Warren (29), with crystalline N-acetyl neuraminic acid (NANA; Sigma Chemical Co.) as standard.

**Paper chromatography.** Acid hydrolysates of neutral and amino sugars were dried in vacuo over P₂O₅ and NaOH, separated on Whatman no. 1 paper by the ascending method with n-butyl alcohol-pyridine-water (6:4:3) or pyridine-ethyl acetate-acetic acid-water (5:5:1:3), and detected with aniline diphensylamine, Morgan-Elson, or 1% ninhydrin spray reagents. Sialic acids were detected as previously described (24).

**Gas-liquid chromatography.** Gas-liquid chromatography was done on a Barber Colman model 5000 chromatograph equipped with a hydrogen flame ionization detector, electrometer with 10⁻¹⁰ amp sensitivity, Honeywell recorder, and disc integrator. Unless otherwise stated, determinations were done isothermally at an oven temperature of 160 C with a...
nition carrier gas flow of 45 ml/min, on 6-ft (1.8-
 meter) U-shaped glass columns of 1% (w/w) OV-17
Polymer on 100 to 120 mesh Gas Chrom-Q (Regis
Chemical). The O-trimethylsilyl ether derivatives of
both samples and standards were prepared by the
method of Sweedy and Walker (25). The methanalysis
conditions for both 2- to 4-mg samples and standards
were 2 N methanolic HCl for 6 to 24 hr at 100 C. Hy-
drosylates were passed over amberlite CG-45 and
Dowex 50W-X4 (to remove amino sugars) columns
(10 by 1 cm) and dried under N2 prior to silylation.
Trimethylsilyl ether derivatives of group C hydro-
sylates (0.5 N methanolic HCl, 80 C, 60 min) for detec-
tion of sialic acids were chromatographed on 10%-
SE-30 on 80 to 100 mesh Gas Chrom-S columns with
the use of a programmed temperature increase of 2.0
C/min (180 to 260 C) and were compared with authen-
tic NANA under identical conditions.

The methyl glycosides were identified both by rela-
tive retention times with reference to an internal
standard (L-fucose) and designation of an arbitrary
value of 1.0 to α-glucose, and by co-chromatography
with standards derived under identical conditions. In-
jection sample volume was 1 to 4 μl.

Infrared absorption spectra. Infrared absorption
spectra were determined with a Beckman IR-9 spec-
trophotometer by use of 1.5-mg samples of standards
or antigen pressed into potassium bromide discs and
scanned at 80 cm⁻¹ for 3.2 min.

Enzyme studies. Group C antigen samples were hy-
drolyzed with Clostridium perfringens neuraminidase
(type V, Sigma Chemical Co.), with an activity of
0.08 unit/mg of α-acetyl neuraminic-lactose substrate.
Antigen samples (500 μg) were incubated at pH 5.2
for 48 hr at 37 C with a total of 0.08 unit of enzyme
and were assayed for free sialic acid by the thiobarbi-
turic acid (TBA) technique.

Inhibition studies. Studies were undertaken in an
attempt to inhibit interaction between the group C
and Y antigens and their specific antisera by simple
and substituted sugars in immunodiffusion (16) and
hemagglutination inhibition (10) systems. The tech-
nique used was a modification of the method of
Ullman and Cameron (26) and employed crystalline
NANA, glucose, fructose, mannose, sucrose, glucosa-
mine, galactose, galactosamine, and glucuronic acid
in concentrations up to 5 mg/ml as inhibitors.

Sedimentation velocity. Sedimentation velocity stu-
dies for calculation of s₂₀,₅₅ were performed by use of a
model E ultracentrifuge with schlieren optics (5). Sam-
ple were centrifuged at 20,136 × g at a constant
temperature of 20 C in phosphate-buffered saline,
pH 7.2 (PBS). Plates were measured by use of a two-
dimensional Gaertner microcomparator. Partial spe-
cific volumes and viscosities of both antigens were
measured at 15, 20, and 25 C at concentrations of 1, 4,
and 8 mg/ml in PBS (22).

Molecular weights. Molecular weights were deter-
mined by the sedimentation equilibrium method of
Lamm (14) with the use of schlieren optics and a short-
column equilibrium cell. The schlieren phase plate
angle was 80°. Rotor speeds of 10,589 and 11,272
rev/min were used at a temperature of 20 C. The
TABLE 1. Chemical composition of Neisseria meningitidis group C and Y polysaccharide antigens

<table>
<thead>
<tr>
<th>Component</th>
<th>Amt (μg/100 μg of dry antigen)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Group C</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.6</td>
</tr>
<tr>
<td>Mannose</td>
<td>2.0</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>4.2-7.3</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>70.0</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>5.34</td>
</tr>
</tbody>
</table>

* Expressed as free base.
* Expressed as N-acetyl neuraminic acid.
* Not present.
* Total nitrogen by pyrolysis.

TBA-reactive material regardless of the group C strain studied. However, C. perfringens neuraminidase cleavage of the same group C strains yielded 70% TBA-reactive material. Total neuraminic acid content was identical in all group C strains studied and absent in all group Y strains by both resorcinol and TBA techniques, indicating the lack of substituted NANA or other 2-keto-3-deoxy-sugars in either antigen. TBA-reactive material was identified as NANA by elution at 245 C by programmed gas-liquid chromatography on 10% SE-30 and by migration against NANA standards on paper chromatography. Infrared spectroscopy of two group C strain antigens and a crystalline NANA standard are shown in Fig. 2. The marked similarity of the two antigens to the NANA standard is apparent. In addition, three other group C antigens had similar spectra. The infrared absorption spectrum of group Y antigens reveals strong banding at 2,940 cm⁻¹ (assignment-CH₂), suggesting repetition of a single-CH₂-configuration in polymer form.

Neuraminidase cleavage of the group C antigen for 48 hr abolished the antigen's ability to inhibit the hemagglutination of group C sensitized sheep erythrocytes. Crystalline NANA or glucosamine at concentrations up to 5 mg/ml did not inhibit hemagglutination with either antigen. Similarly, mannosamine, glucuronic acid, galactosamine, glucosamine, and their parent hexoses, as well as sucrose and fructose in the same concentrations, did not inhibit hemagglutination of the group C and Y sensitized sheep erythrocytes. Immunodiffusion studies done with 1% agar gels containing the same sugar concentrations did not inhibit specific immunoprecipitation of either antigen.

Physical studies. The pI of the group C and Y antigens was 1.9 and 2.4, respectively. Figure 3 shows the results of typical elution patterns of the group C and Y antigens after electrofocusing determined by hemagglutination. Studies were also done in which immunodiffusion and TBA reactivity were used to localize the group C peak, and these coincided with the hemagglutination peaks. The group Y antigen precipitated at the point of polymer electroneutrality. Colorimetric analysis by the Elson-Morgan test for hexosamine was positive, and the amino sugar was definitively identified as glucosamine by paper chromatography. The dry weight of the precipitate accounted for 95% of the antigen applied to the column.
Studies of partial specific volumes indicated that at 4 mg/ml and 20°C the partial specific volume of the group C antigen was 0.76 and of the group Y antigen was 0.69. In sedimentation velocity experiments, each antigen sedimented as a single homogeneous peak. The $s_{20w}$ for the group C antigen was 3.36, and for the group Y antigen was 3.84. Molecular weights of both antigens were determined at two rotor speeds. The average molecular weight of the group C antigen was 88,000, and that of the group Y antigen was 197,000. Analytic acrylamide gel electrophoresis confirmed this difference in molecular size, as noted by the greater migration of the group C antigen into 4% acrylamide gels (Fig. 4). In addition, the group C antigen demonstrated banding in 5, 6, and 7% gels, whereas the group Y antigen was excluded from 6 and 7% gels.

**DISCUSSION**

The study of acidic polysaccharides in recent years has revealed their widespread presence in nature and their potential biological importance (13). The meningococcal group C and Y antigens are acidic polysaccharide polymers. The group C antigen contains predominately $n$-acetyl neuraminic acid with small amounts of glucose, glucosamine, and mannose. The use of gas-liquid chromatography with its high degree of sensitivity has allowed detection of small amounts of neutral sugars not previously found in this antigen. Watson (30) also detected hexosamine in his group C preparation, and Gotschlich (11) found only $n$-acetyl neuraminic acid. The group C antigen, despite the presence of neutral and amino sugars, was susceptible to neuraminidase digestion which destroyed the antigenic activity of the preparation. This would indicate that the neutral and amino sugars are present either terminally or as side chains to the repeating units of $n$-acetyl neuraminic acid. The pI of this antigen indicates a high degree of negative charge which is due to the numerous polar groups on the $n$-acetyl neuraminic acid molecule. These molecule elute at the void volume of G-200 despite molecular weights of 88,000. This may be attributed to the hydrophilic character of the carboxyl group which creates hydration shells around the molecule, excluding it from the molecular sieve (2).

The group Y antigen is unique because of the large amount of glucose present in the molecule. It also contains glucosamine and mannose but is devoid of $n$-acetyl neuraminic acid by both TBA and resorcinol reactivity, by paper chromatography, and by gas-liquid chromatography. It has a
high negative charge as demonstrated by electrofocusing and is excluded from G-200 despite a molecular weight of 197,000, probably by the same phenomena discussed above for the group C antigen. The large amount of glucose in this antigen preparation gave us concern that we might be hydrolyzing dextran eluting from the G-200 itself. Colorimetric and gas chromatographic studies of G-200 column blanks failed to detect any neutral or substituted sugar. Neutral and substituted sugars did not inhibit the interaction of these antigens and their specific antibody. These results are similar to those of Mergenhagen (15) and suggest that either chain length of the polymer or some configurational site, or a combination of both, is important in maintaining the antigenic determinant.

Because of the absence of heptoses and keto-deoxy-octonic sugars in these preparations, it is unlikely that they contain endotoxin. Studies which will be published shortly indicate these preparations have unique biological effects distinct from those of endotoxin.

ACKNOWLEDGMENTS

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LITERATURE CITED


FIG. 4. Acrylamide gel electrophoresis of the group C and Y antigens into 4% acrylamide-0.2% bis-acrylamide gels.