Carbohydrate-Containing Group-Specific Antigens of *Neisseria meningitidis*[1] (34452)

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(Introduced by Dr. J. Holper)

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Antigens have been isolated from *Neisseria meningitidis* which are group-specific by passive hemagglutination (PHA) and genus-specific by complement fixation (CF) (1, 2). Hemagglutination-inhibition (HI) studies confirm the high degree of specificity of these antigens (3). No previous attempt has been undertaken to chemically describe these antigens.

A unique property of bacterial antigen adsorption on unmodified erythrocytes (RBC) and the subsequent agglutination of such antigen-treated RBC by specific antibody was first reported in 1948 by Keogh *et al.* (4), and later by Neter *et al.* (5). This adsorption was apparently enhanced by first modifying the antigen with heat treatment or by mild alkaline hydrolysis. The exact role that such treatment plays in adsorption is not fully understood. The fact that they adsorbed to an unmodified RBC suggested that the antigen was probably a polysaccharide. This was in contrast to cell sap *N. meningitidis* antigens of Sanborn and Vedros (6) in which the RBC were modified with tannic acid before sensitization.

This paper deals with the chemical identity and size of the group-specific hemagglutination (HA) antigen of *N. meningitidis*. In addition, the minimal quantity of polysaccharide required to sensitize RBC was determined for each antigen preparation.

Materials and Methods. Crude antigens to various groups of *N. meningitidis* were prepared by methods of Edwards and Driscoll (1) and further purified by the method and preparation of polysaccharide antigens as described by Westphal *et al.* (7), modified as follows: 1 vol of the aqueous portion following phenol extraction was dialyzed in tap water and added, with constant stirring, to 3 vol of cold acetone. The precipitated polysaccharides were then separated in a Spinco model L centrifuge at 8700*g* for 10 min. These included antigens to stock cultures of groups A (MK04), B (6B6), C (PTS-5), Bo (Boshard), and 29E.

Serological titration. Complement fixation (CF) and HA tests were performed by the methods outlined by Edwards *et al.* (1, 2) on the crude antigens and preparations following further purification. Immune sera were prepared in this laboratory using the procedure of Edwards and Devine (2).

Enzymatic treatment. Ten-mg quantities of crude antigens were digested with either 1.0 ml of 0.7% (w/v) solutions of ribonuclease (RNase)[2] (essentially salt free—no protease) in buffered saline, or with 1.0 ml of 0.1% (w/v) solutions of deoxyribonuclease (DNase)[2] (IX crystallized) in buffered saline. Digestion was carried out for 2 hr at 35°, pH 7.8.

Crude antigens (10 mg/ml) were also

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[2] Sigma Chemical Co., 3500 DeKalb St., St. Louis, Mo.

TABLE I. Antigen Titration and Chemical Composition of the Crude and Purified Acetone Insoluble (Fraction) to 5 Groups of *N. meningitidis.*[a]

N. meningitidis antigen, 10 mg/ml	Crude antigen (mg/ml)			Crude antigen (titer reciprocal)		Purified antigen (mg/ml)			Purified antigen (titer reciprocal)	
	Protein	DNA	CHO	CF	HA	Protein	DNA	CHO	CF	HA
A	5.15	1.5	0.87	64	512	—	—	0.73	32	256
B	4.75	2.27	0.59	128	16	—	—	0.51	16	16
C	3.95	0.86	0.63	128	512	0.25	—	0.59	64	256
Bo	4.75	0.46	0.48	128	2048	—	—	0.44	64	512
29E	5.15	0.72	0.45	128	2048	0.10	—	0.50	128	2048

[a] DNA = deoxyribonucleic acid; CHO = carbohydrate; CF = complement fixation; and HA = hemagglutination.

treated with 2.5% (w/v solution of trypsin (1:250) in buffered saline, sterilized by filtration through glass, then diluted 1:10 in buffered saline prior to use. One ml of the crude antigens was adjusted to pH 9.0 and digested for 24 hr at 36° with 1.0 ml of the trypsin solution.

Analytical measurements. Chemical analysis of the crude and partially purified preparations included the Lowry method for pro-

TABLE II. Dilution of Carbohydrate-Containing Group-Specific Antigen of *N. meningitidis* for the Sensitization of RBC.

	Group of *N. meningitidis*									
	A		B		C		Bo		29E	
Antigen dilution	(μg of CHO/ml)	HA	(μg of CHO/ml)	HA	(μg of CHO/ml)	HA	(μg of CHO/ml)	HA	(μg of CHO/ml)	HA
1:5	37.0	128	—	—	39.0	4096	—	—	20.0	256
1:10	18.5	256	150	128	19.5	512	154	2048	*10.0*	*128*
1:20	9.25	256	75	128	9.7	256	77	2048	5.0	4
1:30	*6.2*	*256*	—	—	*6.55*	*256*	—	—	—	—
1:40	—	—	*37.5*	*128*	—	—	38	2048	—	—
1:50	3.7	4	—	—	3.9	32	—	—	—	—
1:80	—	—	8.75	32	—	—	19	2048	—	—
1:100	1.85	2	—	—	1.95	—	—	—	—	—
1:160	—	—	9.4	32	—	—	10	2048	—	—
1:320	1.1	2	4.7	16	—	—	5	2048	—	—
1:640	—	—	2.35	16	—	—	2.5	2048	—	—
1:1280	—	—	1.27	16	—	—	1.25	1024	—	—
1:2560	—	—	0.63	4	—	—	0.65	1024	—	—
1:5120	—	—	—	—	—	—	0.325	1024	—	—
1:10,240	—	—	—	—	—	—	0.165	256	—	—
1:20,480	—	—	—	—	—	—	*0.08*	*256*	—	—
1:40,960	—	—	—	—	—	—	0.04	64	—	—
1:81,920	—	—	—	—	—	—	0.02	32	—	—
1:163,840	—	—	—	—	—	—	0.01	16	—	—
Total (μg/CHO/ml)	188		1500		195		1540		162	

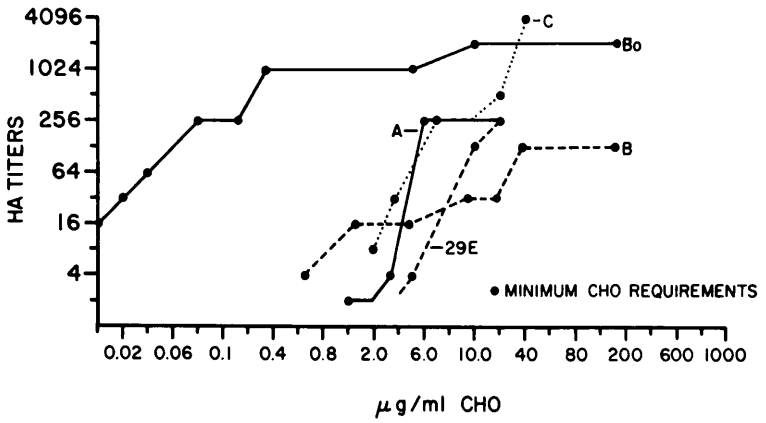

FIG. 1. Minimum carbohydrate antigens to 5 groups of *N. meningitidis* required for sensitization of RBC.

teins (8), the detection of deoxyribonucleic acid (DNA) (corrected for sugar other than 2-deoxyribose) (9, 10), ribonucleic acid (RNA) by the orcinol method (11), assay for sialic acid (12) and carbohydrate (13). These determinations were made using a Beckman model DU spectrophotometer. For further characterization, 0.5 ml of group-specific antigen was mixed with 0.5 ml of a 0.5% blue dextran ($\overline{M}_w$ 2,000,000) and applied to a column 1 × 30 cm containing Sephadex G-200 equilibrated with 0.1 *M* phosphate buffer, pH 7.2. One-half-ml fractions were collected to determine the HA activity (1) and dye transmittance at 540 mμ. A Spinco model E analytical centrifuge was used for the determination of the molecular weight of the group-specific antigen.

Results. The crude and partially purified antigens resulted in the composition shown in Table I. It is apparent that the major part of the crude antigen is protein with no detectable RNA or sialic acid. After the rather simple purification step of phenol extraction followed by precipitation with 3 vol of cold acetone, all of the DNA and most of the protein was removed without altering the carbohydrate concentration. Furthermore, as a result of purification, no significant diminution was detectable in the CF and HA activity. Neither the RNase, DNase, or trypsin digestion diminished the HA activity of the crude or purified antigens.

Antigen dilution for maximum sensitization of RBC as related to carbohydrate concentration is shown in Table II. The evaluation of HA titers of >16 as an end point shows antigen dilution resulting in carbohydrate concentrations of 4–10 μg/ml, in which 2 vol were used to sensitize 1 vol of 2% RBC, with antigens to groups A, B, C, and 29E; whereas, considerably less (0.02 μg/ml) was needed to sensitize against the Bo antigen. Furthermore, if HA titers of 128 or greater are selected as the end point, then as shown, the carbohydrate concentration requirements of antigens to groups A, C, and 29E were 6–10 μg/ml, and 25 μg/ml to the group B antigen; whereas, considerably less carbohydrate (0.08 μg/ml) to the Bo antigen was required for the same degree of sensitization.

The minimum carbohydrate antigen (μg/ml) required (lowest concentration for a given HA titer) for sensitization of RBC is shown in Fig. 1. A difference of 0.3 μg/ml of Bo carbohydrate antigen resulted in a 6-tube difference in titers (16–1024); whereas, from 4 to 6 logs less HA activity occurred with similar levels of group B carbohydrate antigen. The sensitization curves for carbohydrate antigen to groups A, C, and 29E were more closely related and appeared to fall between these two extreme curves. The HA activity of the group-specific antigen, as shown in Fig. 2, indicates that the size of the carbohydrate molecule must be similar to or greater than the exclusion limit of 200,000 for the gel particle, since both the blue dextran 2000

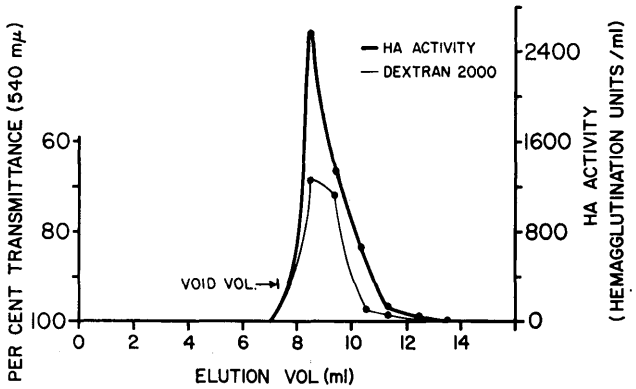

FIG. 2. Elution profiles of blue dextran 2000 and *N. meningitidis* group A HA antigen [antigen preparation: Edwards and Driscoll, Proc. Soc. Exp. Biol. Med. 126, 876 (1967) (1)] using Sephadex G-200, 0.01 *M* PBS, pH 7.2.

used as a marker and the HA activity appeared within the void volume. As shown in Fig. 3, molecular weights, of 83,416 and 170,500 (mean 140,000) were determined by sedimentation equilibrium for the HA antigen to group A *N. meningitidis*.

Discussion. Group-specific antigens to *N. meningitidis* prepared by methods outlined by Edwards and Driscoll (1) contained protein, nucleic acid, and carbohydrate. Crude antigens to 5 groups of *N. meningitidis* were purified and found to be essentially free of protein and nucleic acid, while carbohydrate concentration remained unchanged (0.4–0.9 mg/ml). No sialic acid was detected. Purification did not appreciably diminish either CF or HA activity. The question of whether the combining sites of the antigen configuration responsible for CF and HA activity may be on the same molecule remains unresolved. As might be expected from the chemical findings, enzymatic digestion had no effect upon the HA antigen activity. Satisfactory sensitization (as reflected by HA titer of 128 or greater) required 6–10 μg/ml of carbohydrate for groups A, C, and 29E. For some, as yet, unexplained reason, the Bo system requires far less carbohydrate (0.08 μg/ml, HA titer of 256), while group B needed as much as 25 μg/ml, HA titer of 128, for satisfactory sensitization. This difference in quantity of carbohydrate required to satisfactorily sensitize a sheep erythrocyte for hemagglutination is even more marked when using human antisera rather than hyperimmune rabbit antisera for determining optimal amounts of carbohydrate for sensitization. Antigens prepared from *N. meningitidis* groups A, C, Bo, and 29E appear to sensitize the erythrocyte equally for hemagglutination with either rabbit or human antisera. However, it requires 8 times the amount of group B antigen to sensitize an erythrocyte to agglutinate with human antisera as it does with rabbit antisera (14). These differences in the amount of group-specific antigen required for the sensitization of RBC may result from the configuration and number of available antigenic determinants of the polysaccharide, as to combinations of hexose units.

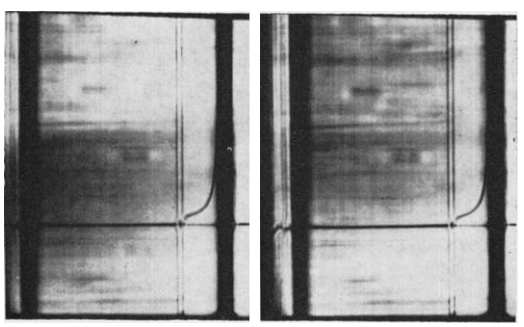

FIG. 3. Molecular weights of 83,410 and 170,500 (mean 140,000) were determined by sedimentation equilibrium for the group A specific antigen to *N. meningitidis*. Photographs at 30 and 36 hr were taken after centrifugation at a speed of 6000 rpm at 18.5°. The centrifugal direction is toward the right. Heparin was used as a standard, $\bar{v}$ 0.47, in a double sector cell.

Summary. A bacterial carbohydrate-containing group-specific antigen of *N. meningitidis* has been identified to be of a high molecular weight (mean 140,000) by sedimentation equilibration and greater than 200,000 by Sephadex G-200. This antigen was proved to be of serological importance for the measurement of antibody by PHA technique. Enzymatic digestion, with RNase, DNase, and trypsin, along with selective methods of isolating polysaccharides, was performed without appreciably diminishing the HA activity. Satisfactory levels of carbohydrate for sensitization of RBC were from 6 to 10 μg/ml (A, C, and 29E), 25 μg/ml (B) and 0.08 μg/ml (Bo). The concentration requirements of the group-specific carbohydrates were discussed along with the possibility of a multivalent molecule with both CF and HA activity and the likely difference in size of antigenic determinants.

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