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MENINGOCOCCAL POLYSACCHARIDE VACCINES

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ABSTRACT OF THE DISCLOSURE

This disclosure describes a process for preparing and isolating antigenic group-specific polysaccharides of serologic group A and group C meningococci used as vaccines. The process employs a cationic substance, such as hexadecyl trimethylammonium bromide, to precipitate the polysaccharide from the whole culture. The antigenic polysaccharides isolated by this process are of high molecular weight (molecular weight 60,000–5,000,000). The main constituent of the antigenic group A polysaccharide is N-acetyl, O-acetyl mannosamine phosphate and the main constituent of the antigenic group C polysaccharide is N-acetyl, O-acetyl neuraminic acid. The two antigenic polysaccharides are useful for producing immunity against meningococcal meningitis in humans.

The invention described herein may be manufactured and used by or for the Government for governmental purposes without the payment to me of any royalty thereon.

BACKGROUND OF THE INVENTION

(1) Field of the invention

This invention is in the field of vaccines for immunization against meningococcal meningitis. More specifically, this invention relates to a new process for isolating antigenic, high molecular weight (molecular weight 60,000–5,000,000), polysaccharides from meningococcus cultures. This invention also includes two new meningococcal vaccines comprising two new antigenic high molecular weight (molecular weight 60,000–5,000,000) polysaccharides from meningococcus cultures.

(2) Description of the prior art

Meningococcal meningitis annually attacks between 200 and 500 U.S. military personnel and between 2,500 and 3,000 civilians in the United States. The mortality rate from this disease averages 7 to 10% in the afflicted military personnel and 26 to 29% among the afflicted civilians. In past epidemics in the United States, up to 30,000 people have been afflicted with a resultant mortality rate of up to 30%.

Meningococcal meningitis is usually caused by the species *Neisseria meningitides*. The species has been subdivided by serological methods and the present classification includes serologic groups A, B, C and D. See S. Branham, Int. Bull. Bacteriol. Nomencl. Taxon., 8:1 (1958). Each of these serogroups can be identified by the characteristic capsular polysaccharide associated with that particular group. Group A meningococcus has been responsible for the majority of meningococcal epidemics and this organism represents a major public health hazard now that sulfonamide-resistant strains have been isolated overseas. See C. Alexander et al., Science, 161:1019 (1968).

More than twenty large scale attempts have been made to develop a vaccine to combat meningococcal meningitis. Several of the vaccines were clearly ineffective, while others yielded inconclusive results. A vaccine produced at the Pasteur Institute in Brazzaville from 1936 to 1939 was apparently successful. Unfortunately, the material produced by the same institute after World War II was no longer effective. See L. Lapeyssonie, Bull. World Health Organ., 28 (Suppl.):1 (1963).

The study of immunity to systemic meningococcal disease has been greatly complicated by the lack of a satisfactory animal model. Mucin-enhanced infection, albeit unsatisfactory, is the only animal model presently available. See P. Miller, Science, 78:340 (1933). The studies with hyperimmune sera of several animal species and convalescent sera of man indicated that antibodies to the group-specific polysaccharides were able to passively protect mice against fatal meningococcal infection. See H. Scherp et al., J. Exp. Med., 81:85 (1945), R. Watson et al., J. Immunol., 81:331 (1958) and E. Kabat et al., J. Exp. Med., 81:1 (1945). But when Kabat et al. investigated the immunogenicity of the group A polysaccharide in human volunteers, they found it to be a poor antigen. See J. Exp. Med., 80:299 (1954). Kabat also injected several human volunteers with a group C polysaccharide prepared according to the method of R. Watson et al., supra, but again the results were disappointing. See E. Kabat, in Polysaccharides in Biology, G. F. Springer, editor. Josiah Macy, Jr. Foundation, New York, N.Y. (1958).

In fully grown *Neisseria meningitides* cultures, the group A or group C-specific polysaccharides are found in great part in the culture supernate, and this may be related to the autolytic properties of the species. Therefore in the prior art, these polysaccharides have always been isolated from culture fluids concentrated either by ultrafiltration or rotary evaporation. See H. Scherp et al., J. Exp. Med., 61:753 (1935), E. Kabat et al., J. Exp. Med., 80:299 (1945), and R. Watson et al., J. Immunol., 81:331 (1958). Significantly, Mergenhagen et al. have shown that group A or C polysaccharides prepared according to the above-described prior art methods have molecular weight *below 50,000*. See J. Immunol., 90:312 (1963).

SUMMARY OF THE INVENTION

This invention comprises a process for the preparation and isolation of antigenic meningococcal polysaccharide with a molecular weight of 60,000–5,000,000 by growing in a suitable medium a culture of serologic group A or group C meningococci and isolating the polysaccharide from the culture by adding a cationic substance whereby the cationic substance and the polysaccharide form a complex which is isolable and then dissociating the cationic substance and the polysaccharide.

This invention further comprises a composition of matter useful for producing immunity against serologic group A meningococcal meningitis which comprises an antigenic polysaccharide with a molceular weight of 60,000–5,000,000 whose main constituent is N-acetyl, O-acetyl mannosamine phosphate.

This invention also comprises a composition of matter useful for producing immunity against serologic group C meningococcal meningitis which comprises an antigenic polysaccharide with a molecular weight of 60,000–5,000,000 whose main constituent is N-acetyl, O-acetyl neuraminic acid.

This invention also includes a meningococcal meningitis vaccine comprising an effective amount of an isotonic saline solution containing an antigenic polysaccharide with a molecular weight of 60,000–5,000,000 whose main constituent is N-acetyl, O-acetyl mannosamine phosphate.

This invention also includes a meningococcal meningitis vaccine comprising an effective amount of an isotonic saline solution containing an antigenic polysaccharide with a molecular weight of 60,000–5,000,000 whose main constituent is N-acetyl, O-acetyl neuraminic acid.

Accordingly, it is an object of this invention to provide

a process which will produce an antigen which is successful in producing immunity against meningococcal meningitis.

Another object of this invention is to provide a process which will produce antigenic high molecular weight polysaccharides which are successful in producing immunity against meningococcal meningitis.

A further object of this invention is to provide compositions of matter which will be successful in producing immunity against meningococcal meningitis.

A further object of this invention is to provide a composition of matter useful for producing immunity against serologic group A meningococcal meningitis which comprises an antigenic high molecular weight polysaccharide.

Finally, it is an object of this invention to provide a composition of matter useful for producing immunity against serologic group C meningococcal meningitis which comprises an antigenic high molecular weight polysaccharide.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The process—Description of the process of preparing antigenic high molecular weight meningococcal polysaccharides

This is a new process for the isolation of the antigenic meningococcal group-specific polysaccharides. With this procedure, it is possible to produce highly purified group A and group C polysaccharides having molecular weights of 60,000 to 5,000,000.

Meningococcal strains: The majority of the strains used came from the collection of the Department of Bacteriology, Walter Reed Army Institute of Research. All lots of group A polysaccharide were prepared from cultures of strain A1, isolated from spinal fluid in Germany in 1965. All lots of C palysaccharide were prepared from strain C11 which was also isolated from spinal fluid in Germany in 1965.

Media and cultural conditions: Mass cultures were performed in the casamino acid medium described by R. Watson et al. in J. Immunol., 81: 331 (1958). Certified casamino acids (commercially available) were substituted for technical grade material. Other fluid media used were the defined medium described by I. Frantz in J. Bacteriol., 43: 757 (1942) and a medium which consisted of a 1/5 dilution of medium 199 and a 1/10 dilution of the casamino acid medium described by R. Watson et al., supra.

The organisms, which were preserved either in lyophile or by freezing in a medium consisting of 5% w./v. bovine serum albumin and 5% w./v. monosodium glutamate, were streaked onto Mueller-Hinton agar and incubated at 37° C. in a candle jar. The organisms were taken from the agar with sterile cotton swabs, suspended in 50 ml. of fluid medium in a 250 ml. conical flask, and incubated at 37° C. for three hours on a rotary platform shaker revolving at about 120 r.p.m. A 5 ml. portion of this culture was transferred into another 50 ml. aliquot of fluid medium and allowed to incubate as described for four hours. This culture was poured into either 500 ml. of media in a 2000 ml. conical flask or 1000 ml. of media in a 4000 ml. flask which was incubated as described until ready for harvesting. The doubling time of meningococci grown under these conditions was approximately one hour and the final bacterial density was about 2×10^9 organisms/ml. Many attempts were made to grow meningococci in 30 liter lots of media contained in 40 liter carboys. The inoculum consisted of 1000 ml. of an actively growing culture, and aeration was provided by bubbling filtered air. Except in one instance, the organisms grew poorly or not at all under these conditions.

Chemical Reagents: Technical grade hexadecyl trimethylammonium bromide (commercially available) and chloroform N.F. (commercially available) were used. All other chemicals employed were reagent grade.

Preparation of group-specific meningococcal polysaccharides: The group-specific polysaccharides do not remain cell-associated in a fully grown culture, and it is therefore necessary to isolate the polysaccharides from the culture fluid. The method of isolation employs a cationic substance, for example, the detergent hexadecyl trimethylammonium bromide, to rapidly precipitate the polysaccharides from the whole culture. The group A and C polysaccharides are negatively charged and the cationic substance complexes with the polysaccharide to cause precipitation.

After the purity of each culture was established by examination of a gram-stained smear, a 10% w./v. solution of hexadecyl trimethylammonium bromide was added to a final concentration of 0.10% w./v. The precipitate was collected by centrifugation (20,000 g. for five minutes), the supernate being discarded. The precipitate was thoroughly homogenized with 150 ml. of water in a mixer and then centrifuged for 10 minutes at 13,000 g.

Dissociation of the complex: The precipitate was extracted three or four times with 100 ml. aliquots of 0.9 M calcium chloride in a mixer and centrifuged for 15 minutes at 13,000 g. after each extraction.

Removal of nucleic acids: Absolute ethanol was added to a final concentration of 25% v./v. to the pooled slightly cloudy or opalescent supernates of the calcium chloride extractions. A fibrous precipitate consisting of nucleic acid formed immediately and was removed by spooling onto a glass rod. The remaining suspension was placed at 4° C. for three hours and centrifuged at 2° C. at 13,000 g. for 15 minutes. This yielded a perfectly clear supernate to which absolute ethanol was added to a final concentration of 80% v./v., causing the precipitation of the polysaccharide. However, the hexadecyl trimethylammonium bromide and the calcium chloride remained in solution, as they are freely soluble in ethanol. This suspension was centrifuged for 10 minutes at 2,000 g. and the precipitate was washed three times with absolute ethanol, twice with acetone, twice with diethyl ether, and dried in vacuo. In some instances centrifugation for 10 minutes at 13,000 g. was necessary to completely sediment the precipitate during the ethanol and acetone washes.

Removal of proteins: The crude polysaccharide was dissolved in 120 ml. of 0.1 M sodium acetate pH 6.8, yielding an opalescent solution which was clarified by centrifugation for two hours at 100,000 g. The clear supernate was drawn off with a Pasteur pipette and homogenized at 0° C. for 30 minutes in a mixer with chloroform and butanol (5:1). The resulting emulsion was centrifuged for 10 minutes at 13,000 g. and the clear aqueous layer was drawn off and again homogenized for one hour. After six cycles of homogenization with chloroform-containing butanol, chloroform alone was used for several further cycles. This procedure was discontinued when the "cake" forming at the interface after centrifugation was almost negligible. Some preparations were further purified by adding to them 2/3 volume of saturated cupric acetate, pH 5.0. The solution was allowed to stand for 10 minutes at room temperature and was then centrifuged for 15 minutes at 13,000 g. The polysaccharide was precipitated with ethanol and converted to the sodium salt by dissolving it in saturated sodium acetate and reprecipitating it with ethanol repeatedly until the blue color was lost. As can be seen in Table I, below, this cupric acetate step is not essential to obtain polysaccharide essentially free of protein or nucleic acid.

Final purification: The polysaccharide was precipitated by the addition of four volumes of ethanol and collected by centrifugation for 10 minutes at 2,000 g. The precipitate was dissolved in 30 ml. of saturated sodium acetate pH 7.0 and reprecipitated with four volumes of ethanol. The material was dissolved in 30 ml. of water and centrifuged for two hours at 100,000 g. The polysaccharide contained in the clear supernate was precipitated by addition of four volumes of ethanol. The precipitate was col-

lected by centrifugation for 10 minutes at 2,000 g., washed three times with 150 ml. aliquots of absolute ethanol, twice with acetone, and vacuum dried.

The purity of the polysaccharides was assayed by estimating to what extent they were contaminated with nucleic acid or with protein. The protein content in the different lots of polysaccharide was determined by the method of Lowry et al. in J. Biol. Chem., 193: 265 (1951) using bovine serum albumin as a standard, and the nucleic acid content was determined by the ultraviolet absorbancy at 260 millimicrons using

$$E^{0.1\%}_{1\ cm.} \text{ of } 20.0$$

Almost all the group A and C polysaccharide preparations contained less than 1% by weight of either nucleic acid or protein. The following table summarizes the different lots of polysaccharides prepared by the process described:

for varying durations at 110° C. The hydrolysates were evaporated to dryness in a rotary evaporator at 40° C. and the residues were dissolved in 30 ml. of the pH 2.2 buffer used with the amino acid analyzer. The solutions were filtered through millipore membranes and 1.0 ml. portions were analyzed.

For the quantitative determination of sialic acid, 2 to 3 mg. samples of group C polysaccharides were dissolved in 1.0 ml. of water, 200 microliter aliquots were placed into four tubes equipped with polytetrafluoroethylene coated screw caps, and the contents of the tubes were dried over P_2O_5 in vacuo. Hydrolysis of the samples was performed at 65° C. with 500 microliter of methanolic-HCl (3M) for varying intervals of time. The hydrolysates were freed of methanolic-HCl with a stream of nitrogen directed at the solution and analyzed on the amino acid analyzer. The constant used for the calculation of sialic

TABLE I.—SUMMARY OF THE LOTS OF POLYSACCHARIDE PREPARED

			Conditions of growth				Yield, mg.	Contamination, percent		Remarks
Lot No.	Strain	Medium	Containers	Age of culture, hours	Total volume, liters	Treated with Cu++		Nucleic acid	Protein	
A-1	A1	Cas [1]	2 and 4 l. flasks	16	16	Yes	179	0.24	[2]	
A-2	A1	Cas	do	16	20	No	163	0.83	0.88	
A-3	A1	Frantz [3]	40 l. carboy	8	30	Yes	74	2.00	0.79	Medium sterilized by Millipore filtration. Culture lightly contaminated.
A-5	A1	Cas	4 l. flasks	16	30	No	175	0.92	0.35	
C-1	C11	Cas	2 l. flask	16	1					Prepared to test the feasibility of the method for group C cultures.
C-2	C11	Cas	2 and 4 l. flasks	16	20	Yes	180	0.60	0.62	
C-3	C11	Cas	40 l. carboy	16	30	Yes	320	0.85	1.22	Only instance in which good growth was obtained in a carboy.
C-4	C11	Cas	do	22	30	No	80	0.41	0.31	Poor growth.
C-5	C11	Cas	do	22	30	Yes	105	0.18	0.39	Mediocre growth.

[1] Cas refers to the casamino acid medium described by R. Watson et al. in J. Immunol., 81: 331 (1958).
[2] Not done.
[3] Frantz refers to the medium described by I. Frantz in J. Bacteriol., 43: 757 (1942).

THE COMPOSITION OF MATTER—DESCRIPTION OF THE ANTIGENIC HIGH MOLECULAR WEIGHT MENINGOCOCCAL POLYSACCHARIDES

(I) Analytical methods

General analytical methods: As mentioned previously, the protein content in the different lots of polysaccharide was determined by the method of Lowry et al. in J. Biol. Chem., 193: 265 (1951) using bovine serum albumin as a standard, and the nucleic acid content was determined by the ultraviolet absorbancy at 260 millimicrons using

$$E^{0.1\%}_{1\ cm.} \text{ of } 20.0$$

Total nitrogen was determined by the method of Kjeldahl; see Aust. J. Chem., 7: 55 (1954). The method of Chen et al. in Anal. Chem., 28: 1756 (1956) was used for the determination of phosphorous. The acetyl content was determined by the method of A. Inglis in Mikrochim. Acta, 2: 228 (1958) and also by the chromic acid oxidation procedure of E. Weisenberger in Mikromchemie, 33: 51 (1947). Sialic acid content in chromatographic eluates was measured by the method of L. Svennerholm, Biochim. Biophys. Acta, 24: 604 (1957). The anthrone method of L. Mokrasch in J. Biol. Chem., 208: 55 (1954) was used to estimate the amount of dextran in gel filtration eluates. Sodium and calcium content of the polysaccharides were determined by atomic absorption spectrophotometry.

Ion-exchange chromatography: The amino acid and amino sugar content of acid hydrolysates was determined by ion-exchange chromatography with the automatic recording equipment described by Spackman et al. in Anal. Chem., 30: 1190 (1958). Samples of the polysaccharides (about 10 mg.) were dissolved in 10 ml. of water containing 1 micromole each of norleucine and alpha-amino-beta-guanidopropionic acid used as internal standards. An aliquot of 1.0 ml. of this solution was mixed with 1.0 ml. of 12 N HCl and hydrolyzed in sealed evacuated glass tubes

acid from the chromatogram was obtained by treating an authentic sample of N-acetyl-neuraminic acid with methanolic-HCl in the same manner as the group specific polysaccharides.

Gas chromatography: Ninhydrin negative components were detected by hydrolyzing samples of 1 mg. with 2 ml. of 0.5 N methanolic-HCl for 22 hours at 65° C. D-mannitol (25 microliter of a 50 mg./100 ml. solution) was added to the samples as an internal standard. After hydrolysis, the contents of the tube were evaporated to dryness at 65° C. under a stream of nitrogen. The hydrolysates were then treated with 2 ml. of a mixture of pyridine, trimethylchlorosilane, and hexamethyl disilazene (5:1:1, 5 v./v.) at 25° C. After 30 minutes, the reagents were removed at 65° C. under a stream of the nitrogen and the residues were extracted with 2.0 ml. of heptane. The heptane layer was centrifuged to remove the insoluble material and was then dried under nitrogen at 65° C. The residue was dissolved in 10 microliter of heptane. For gas chromatographic analysis, 1 microliter of this sample was injected into the column which was approximately 10 feet in length and 0.125 inch in diameter, and packed with Chromsorb W coated with 3% OV-17 polymer. The column was equilibrated at 120° C. in a gas chromatography machine equipped with a flame-ionization detector.

Gel filtration: Gel filtration was performed on Sephadex G200 in a column 1.5 x 80 cm. using a 0.1 M tris(hydromethyl)aminomethane(Tris)-HCl buffer pH 8.0. The column was calibrated with Dextran T 80 (molecular weight 72,000), Dextran T 40 (molecular weight 42,000), and Blue Dextran (molecular weight 2,000,000). Volumes of fractions were estimated gravimetrically using tared tubes.

(II) Chemical composition and molecular weight of the polysaccharides

Chemical composition of group A polysaccharide, Lot A-12: Amino acids and amino sugars were determined by

the ion-exchange chromatography technique, above, and the value for mannosamine phosphate is corrected for losses due to acid hydrolysis. Acetyl, moisture, nitrogen, and phosphorous were determined by the techniques described above. The results of the analysis on Lot A–2 are presented in Table II, below. The major constituents of this polysaccharide are mannosamine phosphate, acetyl, and sodium. It has not been possible as yet to measure the moisture content of this polysaccharide accurately because charring of the material occurs at temperatures as low as 65° C. The loss of moisture under high vacuum at room temperature was 4% of the weight. There are more acetyl groups present than there are amino groups indicating that this polysaccharide contains O-acetyl groups. Over 90% of the weight of this material can be accounted for as the sodium salt of N-acetyl, O-acetyl mannosamine phosphate and moisture. The fact that there is almost one residue each of nitrogen and phosphorus per mole of mannosamine phosphate would suggest that the A-substance isolated by the present procedure is nearly a pure polymer of mannosamine phosphate. It should be noted that the A-substance contains less than 1% by weight of all the amino acids combined and is, therefore, free of protein and mucopeptide. Muramic acid is also absent. The polysaccharide was also examined by gas chromatography. The material contains no glucose, galactose, mannose, xylose, fucose, or ribitol.

TABLE II.—CHEMICAL COMPOSITION OF GROUP A POLYSACCHARIDE, LOT A-2

	Weight per 100 mg. of sample, mg.	Amount per 100 mg. of sample, micromoles
Composition:		
Mannosamine phosphate[1]	67.24	279
Acetyl	13.48	321
Sodium	5.66	246
Moisture	4.00	
Recovery	90.38	
Calcium	<0.15	<5
Total amino acids	<1.00	<10
Nitrogen	3.77	269
Phosphorous	8.23	266

[1] Calculated as mannosamine phosphate-water; molecular weight 241

All the other lots of group A polysaccharide, although not fully analyzed, contained not more than 8% phosphorous indicating they they resembled Lot A–2.

Chemical composition of group C polysaccharide, Lot C–2: The results shown in Table III indicate that the C substance prepared by this procedure is nearly a pure polymer of sialic acid (also known as N-acetyl neuraminic acid). The material contains less than 1% of protein or mucopeptide. Gas chromatography failed to reveal any glucose, galactose, mannose, xylose, fucose, or ribitol. Nearly all the nitrogen in the preparation can be accounted for as sialic acid. In addition to N-acetyl, O-acetyl groups were also found to be present in the group C polysaccharide. Hence, the main constituent of the group C polysaccharide is N-acetyl, O-acetyl neuraminic acid. The moisture content was found to be 9.5%. The results of these analyses indicate that the sum of the moisture, sodium, sialic acid and O-acetyl accounts for 94% of the weight of the material. The sialic acid content was also estimated by gas chromatography yielding the same result as the ion exchange procedure.

In Table III, the ammino acids and amino sugars were determined by the ion-exchange chromatography technique, above, and the value for sialic acid is corrected for losses due to acid hydrolysis. Acetyl, moisture, nitrogen, and phosphorous were determined by the techniques described above. The other lots of group C polysaccharide were not fully analyzed but all contained over 75% of sialic acid as determined by the resorcinol reaction described by L. Svennerholm in Biochim. Biophys. Acta, 24: 604 (1957).

TABLE III.—CHEMICAL COMPOSITION OF GROUP C MENINGOCOCCAL POLYSACCHARIDE

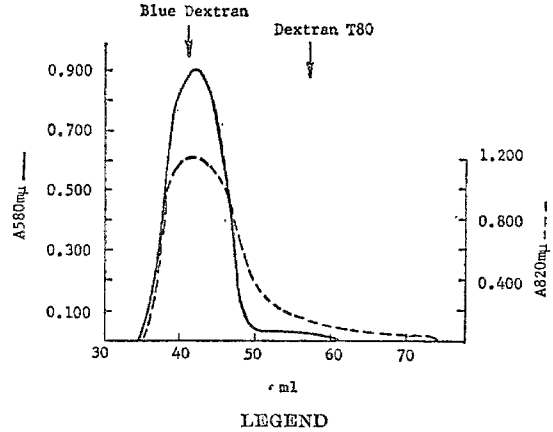
	Weight per 100 mg. of sample, mg.	Amount per 100 mg. of sample, micromoles
Composition:		
Sialic acid[1]	76.24	262
Acetyl[2]	5.80	138
Sodium	2.51	109
Moisture	9.50	
Recovery	94.05	
Calcium	<0.15	<5
Total amino acids	<1.00	<10
Nitrogen	3.81	272
Phosphorus	0.0	0

[1] Calculated as N-acetyl neuraminic acid-H^2O; molecular weight 291.
[2] Only O-acetyl groups are included here. Total acetyl group was 16.81 mg. per 100 mg. or 400 micromoles per 100 mg.

This analysis of the group C polysaccharide differs in one detail from that reported by Watson et al. in J. Immunol., 81: 337 (1958). They found that their preparations of group C polysaccharide contained principally sialic acid but also some hexosamine. Group C polysaccharide has been prepared by methods similar to the ones described by Watson et al. in J. Immunol., 81: 331 (1958), and these preparations contained appreciable quantities of mucopeptide (ca. 15% by weight). This may have been the source of the hexosamine which Watson et al. have described.

Determination of the molecular size of the meningococcal polysaccharides: The molecular size of all the preparations listed in Table I, above, was estimated using Sephadex G–200 gel filtration. The column was calibrated with dextran fractions of known molecular weights. A typical elution profile is shown in FIG. 1 indicating that both the A and C substance eluted as single peaks in the void volume of the column. The void volume was estimated with blue dextran ($\overline{M}w$ 2,000,000) $\overline{M}w$=mean molecular weight) either by separate chromatography or by co-chromatography with the meningococcal polysaccharide. The elution volume of dextran T 80 ($\overline{M}w$ 72,000) is also indicated in FIG. 1. As shown in FIG. 1, the molecular weight of the A and C polysaccharides can range from as low as approximately 60,000 to as high as approximately 5,000,000. The mean molecular weight of the A and C polysaccharides exceeds 100,000. All the preparations of group A or group C polysaccharide listed in Table I behaved the same upon gel filtration indicating that all prepparations had mean molecular weights exceeding 100,000. The elution profile of the group A polysaccharide tended to be skewed as indicated in FIG. 1.

Figure 1



LEGEND

FIG. 1: Gel filtration of group A and C polysaccharide through Sephadex G–200. The solid line is the elution pattern of group C polysaccharide, Lot C–2; the dashed line of group A polysaccharide, Lot A–2. The eluates were monitored either for sialic acid or for phosphorus. Blue

dextran ($\overline{M}w$ 2,000,000) eluated at 41.8 ml. and dextran T 80 ($\overline{M}w$ 72,000) eluted at 57.0 ml.

An important property of the group A and C polysaccharides isolated by my method is that they are of high molecular weight. In this way, they differ from the polysaccharides prepared from culture concentrates. Mergenhagen et al. in J. Immunol., 90: 312 (1963) have shown that the C antigen prepared by Watson et al., above, penetrated Sephadex G–75. Liu et al. (to be published) have shown that both A and C antigens which were prepared from cultures concentrated by rotary evaporation had average molecular weights of less than 50,000. There seems to be no obvious reason why these polysaccharides are intrinsically unstable and probably the depolymerization is enzymatic. My method may circumvent this enzymatic breakdown by the rapidity with which the polysaccharides are isolated from the culture and put into organic solvents. Another possibility is that the cationic substance inactivates the enzymes responsible for the degradation of the polysaccharides.

PROOF OF UTILITY

Serological methods: A hemagglutination technique utilizing polysaccharide coated erythrocytes was developed. Hyperimmune rabbit sera were obtained from the collection of the Department of Bacteriology, Walter Reed Army Institute of Research. Freshly drawn type O Rh-negative human red cells were fixed with glutaraldehyde as described by Bing et al. in Proc. Soc. Exp. Med., 124: 1166 (1967).

Prior to sensitization, the glutaraldehyde fixed red cells were washed 3 times with buffer and diluted to a concentration of 2.5%. Equal amounts of the red cell suspension and buffer containing antigen were mixed and were placed in a 37° C. water bath for 30 minutes. Optimal sensitization was obtained if the antigen solution added to the red cells contained 20 microgram/ml. of A antigen, Lot A–2, or 20 microgram/ml. of C antigen, Lot C–2. After incubation the cells were washed 3 times with buffer and diluted to a red cell concentration of 0.5% with buffer containing 0.05% w./v. bovine serum albumin.

Hemagglutination tests were performed by the microtiter technique employing round bottom plates and calibrated loops and droppers. Phosphate buffered saline pH 7.28 was prepared from $Na_2HPO_4M/15$, $KH_2PO_4M/15$, NaCl 8.2% w./v., and water in the proportions 80:20:100:800. Passive hemagglutination tests to detect antibody were performed by first adding 0.05 ml. of buffer containing 0.5% w./v. of bovine serum albumin to each well. Serial two-fold dilutions of the antisera were carried out with 0.05 ml. loops. The sensitized red cells were then added with a 0.05 ml. dropper. Hemagglutination inhibition (H.I.) tests to measure antigen concentration were performed by adding 0.05 ml. of buffer containing 0.5% w./v. bovine serum albumin to each well, serially diluting the antigens with 0.05 ml. loops and adding 0.025 ml. of antiserum diluted in buffer containing 0.5% w./v. bovine serum albumin. The antiserum was allowed to interact with the antigen for at least 15 min. and excess antibody was then estimated by adding sensitized red cells. The A antiserum #CH1 was diluted 1/1000, and the C antiserum #CH7, 1/1000.

For serogrouping meningococcal isolates by H.I. test, saline extracts of meningococci were prepared by heavily seeding a plate of Mueller-Hinton agar with the strain of meningococcus to be studied. The organisms were harvested from the plate with a cotton swab after 6 to 8 hours of incubation at 37° C. in a candle jar, and were suspended in 1 ml. of phosphate buffered saline. The bacteria were then removed by centrifugation for 10 min. at 30,000 g. and the supernates were stored frozen.

Immunological properties of the group specific polysaccharides: The group A and the group C antigen reacted strongly by capillary precipitation with many homologous meningococcal grouping sera.

Certain preparations of the two polysaccharides were found to readily sensitize glutaraldehyde fixed human type O Rh-neg. red blood cells. The sensitized cells were reacted with several rabbit hyperimmune sera and the results are shown in Table IV.

TABLE IV.—PASSIVE HEMAGGLUTINATION REACTIONS OF RABBIT MENINGOCOCCAL ANTISERA WITH CELLS SENSITIZED WITH THE MENINGOCOCCAL POLYSACCHARIDES

Serum No.	Strain used for immunization	Reciprocal hemagglutination titer with red cells sensitized with— A polysaccharide	C polysaccharide
Group A antisera:			
718	M1027	10,000	[1]—
883	M1027	20,000	—
598	M1027	20,000	—
JE1	M1027	1,280	—
BA	M1027	1,280	—
H10	?	1,280	—
CH1	A1	10,000	—
Group C antisera:			
BC	M1628	—	5,120
JE5	M1054	—	320
CH7	9 Misc.	—	2,560
CH601	9 Misc.	—	2,560
CH568	9 Misc.	—	1,280
27	C11	—	640
30	C11	—	640

[1] A dash indicates that the reciprocal titer was less than 20.

The sera prepared against group A organism (Strain A1 or Strain M1027) reacted with cells sensitized with A polysaccharide but failed to react with cells sensitized with the C antigen. The antisera prepared against 4 group C strains again showed group specificity.

The specificity of the serological reactions was further tested by hemagglutination inhibition. The purified antigens were serially diluted in microtiter plates and appropriate dilutions of rabbit hyperimmune sera directed against groups A and C were added to each well and allowed to interact with the antigen for at least 15 minutes. Excess antibody was then estimated by adding appropriately sensitized red cells to each well. Table V, below, indicates the smallest concentration of each of the polysaccharides capable of completely inhibiting passive hemagglutination.

The sensitivity of this reaction depends upon the dilution of antiserum used in the test. The figures in Table V are primarily intended to illustrate that there is some variability between the different lots of polysaccharide which may be related to differences in molecular size of the antigens.

TABLE V.—COMPARISON OF POLYSACCHARIDE PREPARATIONS FOR THEIR HEMAGGLUTINATION INHIBITION ACTIVITY.

A polysaccharide		C polysaccharide	
Lot A–1	[1] 0.5	Lot C–2	0.5
Lot A–2	0.5	Lot C–3	2.0
Lot A–3	2.5	Lot C–4	0.5
Lot A–5	1.0	Lot C–5	0.5

[1] Concentration in microgram/ml. completely inhibiting passive hemagglutination.

Concentrations as high as 4,000 microgram/ml. of any of these preparations did not inhibit the heterologous reactions. The hemagglutination inhibition test, therefore, proved to be a specific and sensitive test, for the presence of the polysaccharide antigens.

The method is also applicable to crude saline extracts of meningococci. Twelve group A strains, and 25 group C strains were chosen from the collection of The Department of Bacteriology, Walter Reed Army Institute of Research. The strains came from cases of meningococcal disease and from asymptomatic carriers. Furthermore, the strains originated from all areas of the United States, and certain strains were obtained from Europe and North Africa. Saline extracts of these organisms were prepared as described above under "Serological Methods," and these extracts were tested for their ability to inhibit the group A and C passive hemagglutination systems. All group A strains inhibited the A system, but did not inhibit

the group C systems. All group C strains inhibited the group C hemagglutination systems, but not the heterologous system.

Toxicity of the group—Specific meningococcal antigens in laboratory animals: The general safety test employing guinea pigs as described in the Public Health Service Regulations, Title 42, Section 73.72, was performed with 500 microgram doses of meningococcal polysaccharide, Lots C–4, C–5, and A–5. The pyrogenicity of Lots C–4, C–5, and A–5 was tested in albino rabbits (ca. 2.2 kg.) as outlined in the Public Health Service Regulations, Title 42, Section 73.74. Interference with the growth rate of weanling mice was assayed by the method of Pittman et al. in Appl. Microbiol., 13: 447 (1965). The method of Dubos et al. in J. Exp. Med., 113: 921 (1961), was used to assay for the presence of contaminating endotoxin.

(1) Toxicity for guinea pigs: Three guinea pigs weighing ca. 350 gm. were injected with 500 micrograms of each lot of polysaccharide and their weights and temperatures were observed for one week. The lots of polysaccharide C–4, C–5, and A–5 were tested by this method. The animals showed normal weight gain and no febrile responses.

(2) Pyrogenicity in rabbits: A dose of 2.5 microgram of C–4, C–5, or A–5 was injected intravenously into each of 3 albino rabbits. As a positive control other rabbits were injected with 0.010 microgram of endotoxin isolated from the same meningococcal strains (A1 and C11) used for the preparation of the group specific polysaccharides.

The 9 animals injected with the polysaccharide preparations did not exhibit any perceptible pyrogenic responses. However, the 6 animals injected with 0.010 microgram of endotoxin from strain A1 or C11 had definite febrile responses. This experiment indicates that the polysaccharides contained less than 1% by weight of biologically active endotoxin. The specific test data is published in J. Exp. Med., 129: 1371 (1969).

(3) Growth of weanling mice: One of the general toxicity tests employed determined the effect upon the growth rate of weanling mice resulting from the injection with meningococcal polysaccharides. Male or female albino mice weighing 14 to 16 gm. were injected with 100 microgram of the various lots of polysaccharide, and their weights were observed for one week. The experiment was done twice with Lot A–5. The growth rate of the mice injected with polysaccharide was no different than that of animals injected with saline. The specific test data can be seen in J. Exp. Med. 129: 1373 (1969).

(4) Inhibition of drinking by mice: The absence of significant amounts of endotoxin was also demonstrated by the test described by Dubos et al., above. Normal mice gain weight overnight primarily because of water intake. However, animals injected with endotoxin do not drink normally and show a weight loss overnight. Mice were weighed and injected at 4 p.m. with 100 microgram of polysaccharide or with either 1.0 or 0.5 microgram of meningococcal endotoxin. Water and food were supplied ad libitum. The animals were weighed the next morning and the average weight change per mouse was calculated.

The mice injected with polysaccharide generally gained less weight than control animals injected with saline. The injection of one preparation, Lot C–3, caused a loss of weight. However, in no experiment did the mice injected with polysaccharide lose as much weight as the animals injected with 0.5 microgram or 1 microgram of endotoxin. The specific test data is published in J. Exp. Med. 129: 1372 (1969).

Sterility: Sterility of the meningococcal polysaccharide prepared for human use (Lots C–4, C–5, and A–5) was tested in bulk before final packaging and on 10% of the final packaged material, in accordance with the regulations of the Public Health Service, Title 42, Section 73.73.

The immunogenicity of high molecular weight A and C meningococcal polysaccharides in human volunteers: The immunogenicity of the group specific polysaccharides was first tested in one volunteer and then in an additional 5. All of the volunteers were males ranging in age from 24 to 38 years of age, and were laboratory personnel. One of the subjects was a Negro; the others Caucasian. Throat cultures were obtained at the beginning of the study, at weekly intervals for 4 weeks and occasionally thereafter and were examined for the presence of meningococci. All cultures were negative except for subject I.G. who had recently acquired a group B meningococcus. Subject J. W. was known to have carried group C meningococci until about 4 months prior to this study.

The subjects were bled and then injected intradermally in the forearm with 0.20 ml. of isotonic saline containing 50 microgram of polysaccharide. The dates and the sequence of immunizations are indicated in Table VIII, below. The subjects were asked to report any signs or symptoms of toxicity and to measure their temperature 8 hours following the injection. None of the volunteers had any signs of systemic toxicity and no febrile responses occurred.

All subjects showed a skin reaction to the injections. There were no immediate wheal and erythema reactions. About 4 hours following the injection a pale orange discoloration occurred which was limited to the site of the bleb. One subject (E.C.G. injected with A–2) reported mild itching locally which disappeared within 6 hours. At about 8 hours all subjects reported an area of erythema and sometimes slight induration around the site of injection. The reactions ranged up to 50 mm. in diameter, were slightly tender and reached maximum dimensions by 20 hours. They then faded completely over the next 48 hours, leaving in most instances an area of pigmentation about 10 mm. in diameter which disappeared in about 2 weeks.

Serum specimens were collected at weekly intervals for 4 weeks and occasionally thereafter. Antibody responses were measured by several methods.

(1) Hemagglutinating antibodies: Results in Tables VI and VII, below, show that all subjects responded with the production of hemagglutinating antibody within one week of injection of polysaccharide and reached peak titers within 2 weeks. The geometric mean of the reciprocal titers was 161 for the response to the C polysaccharide, and 446 for the response to the A polysaccharide. The titers remained unchanged or dropped one 2-fold dilution during the remainder of the study. Subject J.W. was known to have been a group C carrier until a few months before this study, and this probably accounts for his initial titer of $\frac{1}{32}$ against red cells sensitized with the C antigen. Due to the nature of the hemagglutination system the other initial titers should not be interpreted as indicating either the presence or absence of antibodies. What is of interest are the pronounced increases in titer following the injection of the antigens.

TABLE VI.—RECIPROCAL HEMAGGLUTINATION TITER OF SIX SUBJECTS IMMUNIZED WITH MENINGOCOCCAL C POLYSACCHARIDE [1]

	Weeks following immunization with C antigen							
	0	1	2	3	4	6	20	35
Subjects:								
M.S.A.	2	64	256	256	256	256	512	------
W.C.B.	2	32	256	256	256	------	256	------
I.G.	2	32	128	128	128	128	128	------
E.C.G.	2	128	256	128	------	------	128	128
J.S.	2	64	64	64	64	64	64	------
J.W.	32	64	128	128	128	128	128	------

[1] Cells sensitized with C polysaccharide.

TABLE VII.—RECIPROCAL HEMAGGLUTINATION TITERS OF FIVE SUBJECTS IMMUNIZED WITH MENINGOCOCCAL A POLYSACCHARIDE [1]

	Weeks following immunization with A antigen					
	0	1	2	4	18	37
Subjects:						
M.S.A.	8	128	1,024	1,024	512	------
I.G.	32	128	512	512	256	------
E.C.G.	8	128	512	512	256	256
J.S.	16	64	128	128	64	------
J.W.	16	512	512	512	512	------

[1] Cells sensitized with A polysaccharide.

(2) Precipitating antibodies: The sera of these subjects were also tested qualitatively for the presence of precipitating antibody employing the ring test. All subjects except J.S. had detectable precipitating antibodies to the A and the C polysaccharide within 2 weeks following injection with the respective antigen. The sera of subject J.S. did not at any time produce a positive ring test with either antigen.

(3) Bactericidal antibodies: The bactericidal antibody response resulting from the immunizations was tested by measuring the bactericidal activity of the sera against 4 test strains of meningococci in the presence of an exogenous source of human complement. Two of the test organisms belonged to group A and the other 2 to group C. The results are set forth in Table VIII, below.

All subjects except E.C.G. were injected first with the C polysaccharide. Their bactericidal activities in the group C test organisms rose to full titer within a week or two. Subject J.W. who had been a group C carrier and had a very high initial bactericidal titer to the group C organisms, nevertheless experienced a considerable rise in activity following the injection with C polysaccharide. None of the subjects had increases in bactericidal activity to the group A test organisms as a result of injection with the C polysaccharide, but all volunteers did produce bactericidal antibody to the group A test organisms in response to injection with the A polysaccharide. Subject W.C.B. was not injected with the A polysaccharide and never developed increased bactericidal activity to the group A test organisms. Subject I.G. showed the same pattern of increases in bactericidal response even though he was a carrier of group B meningococci.

Following immunization with the polysaccharide antigens there were demonstrable increases in bactericidal activity to all strains of meningococci belonging to group A or group C. However, no increases in bactericidal activity occurred to organisms belonging to group B or to 135.

TABLE VIII.—SERUM BACTERICIDAL ANTIBODY IN SIX SUBJECTS FOLLOWING IMMUNIZATION WITH MENINGOCOCCAL GROUP SPECIFIC POLYSACCHARIDES

			Reciprocal bactericidal titer against			
			Group A strains		Group C strains	
Subject	Immunogen		A1	121 misc.	C11	107 VI
M.S.A.:						
5 April	C-5	5 April	4	4	16	16
12 April			4	4	128	512
19 April	A-5	19 April	4	4	2,048	1,024
26 April			32	32	2,048	1,024
3 May			128	128	2,048	1,024
13 August			256	256	2,048	2,048
W.C.B.:						
1 April	C-5	5 April	4	4	8	8
12 April			4	4	16	64
18 April			4	4	128	256
26 April			4	4	128	256
3 May			4	8	128	256
13 August			4	4	128	256
I.G.:						
2 April	C-5	5 April	32	32	16	16
12 April			32	16	32	64
19 April	A-5	19 April	32	16	256	512
26 April			32	128	256	256
3 May			64	256	256	512
17 May			64	256	256	512
13 August			128	256	256	512
E.C.G.:						
30 November	A-2	30 November	4	8	8	4
7 December			8	16	8	-------
11 December			64	128	----------	
14 December	C-2	14 December	64	128	8	4
21 December			64	128	256	256
28 December			64	128	256	512
5 January			64	256	256	512
13 August			128	256	128	512
J.S.:						
2 April	C-5	5 April	8	8	4	4
12 April			8	8	64	64
19 April	A-5	19 April	8	16	256	256
26 April			32	64	256	256
3 May			32	128	256	256
17 May			32	64	512	256
26 August			32	32	256	256
J.W.:						
2 April	C-5	5 April	8	8	128	256
12 April			8	8	256	1,024
19 April	A-5	19 April	8	8	1,024	1,024
26 April			128	128	1,024	1,024
3 May			256	256	1,024	1,024
17 May			256	256	1,024	1,024
13 August			256	256	512	512

The immunoglobulin class of the antibodies responsible for the bactericidal activity: Goldschneider et al. in J. Exp. Med., 129: 1327 (1969) have shown that in serum from 2 normal adults the majority of the antibodies bactericidal to meningococci belonged to the immunoglobulin G class. They also demonstrated that the bactericidal spectrum of maternal and cord serum was identical, further evidence that immunoglobulin G antibody is primarily responsible for the meningococcocidal activity, since cord serum contains essentially only this kind of antibody.

Nature of the antibody response to meningococcal group specific polysaccharides. The classes of antibody produced in the blood of the six human volunteers following injection with the meningococcal group specific polysaccharides was studied by indirect immunofluorescence. IgG, LgM, and IgA antibodies were specifically identified using fluorescein conjugated rabbit antisera prepared against the heavy chains of human immunoglobulins. Antibody titrations were done, using group A or group C meningococci, according to the method of Goldschneider et al. in J. Exp. Med. 129: 1307 (1969). The high molecular weight group A and group C meningococcal polysaccharides were excellent immunogens in the six human volunteers. The specific test data appears in J. Exp. Med., 129: 1375–1383 (1969).

Further proof of the immunogencity of high molecular weight A and C meningococcal polysaccharides in human volunteers: Further tests have been performed on human volunteers at Fort Dix, N.J. In the first large scale test, purified meningococcal polysaccharides were administered to 198 men. Each volunteer was injected intradermally with a vaccine comprising 0.2 ml. of isotonic saline containing 50 microgram of either group A polysaccharide Lot A–5 of group C polysaccharide Lot C–5. No adverse reactions were observed in the 145 men who received the group C polysaccharide, nor in the 53 men who were injected with the group A polysaccharide.

As a result of this test hemagglutinating and bactericidal activity developed in the sera of all individuals with the exception of 2 volunteers injected with the group A polysaccharide. During the six week period of observation, the proportion of unvaccinated recruits who acquired group C meningococci in the three companies studied was 38, 42, and 69 percent, respectively. A significantly lower proportion of the individuals vaccinated with group C polysaccharide acquired group C meningococci: 4.6, 24, and 31 percent, respectively. A complete description of the design and results of this test may be found in J. Exp. Med. 129: 1385–1394 (1969).

Another large scale test of the polysaccharides has been conducted. Approximately 14,000 Army volunteers received the group C polysaccharide. Out of the 14,000 men, only five contracted meningococcal meningitis. One of the five cases was caused by the group C organism, and the other four were caused by group B organisms. This is in contrast to a typical sample of 50,000 unimmunized Army recruits. At least 41 cases of meningitis occurred in this sample and 38 of those cases were caused by the group C organism. The specific test data can be seen in New Eng. J. Med., vol. 282, p. 147 (1970).

I claim:

1. A process for the preparation and isolation of antigenic meningococcal polysaccharide with a molecular weight of 60,000–5,000,000 which comprises:

(a) growing in a suitable medium a culture of meningococci;

(b) isolating the polysaccharide from the culture by adding the cationic detergent hexadecyl trimethylammonium bromide whereby the cationic detergent and the polysaccharide form a complex which is isolable from the medium;

(c) dissociating the cationic detergent and the polysaccharide; and

(d) purifying the polysaccharide.

2. The process of claim 1 wherein the meningococci consist of serologic group A meningococci.

3. The process of claim 1 wherein the meningococci consist of serologic group C meningococci.

4. A composition of matter which comprises an antigenic polysaccharide with a molecular weight of 60,000–5,000,000 whose main constituent is N-acetyl, O-acetyl mannosamine phosphate, and which has substantially the following chemical composition expressed in terms of weight per 100 milligrams of sample:

mannosamine phosphate—H_2O, 67.24 milligrams
acetyl, 13.48 milligrams
sodium, 5.66 milligrams
water, 4.00 milligrams
calcium, less than 0.15 milligram
total amino acids, less than 1.00 milligram
nitrogen, 3.77 milligrams
phosphorous, 8.23 milligrams.

5. A composition of matter which comprises an antigenic polysaccharide with a molecular weight of 60,000–5,000,000 whose main constituent is N-acetyl, O-acetyl neuraminic acid, and which has substantially the following chemical composition expressed in terms of weight per 100 milligrams of sample:

N-acetyl neuraminic acid—H_2O, 76.24 milligrams
O-acetyl, 5.80 milligrams
sodium, 2.51 milligrams
water, 9.50 milligrams
calcium, less than 0.15 milligram
total amino acids, less than 1.00 milligram
nitrogen, 3.81 milligrams
phosphorous, 0.0 milligram.

6. A meningococcal meningitis vaccine comprising an isotonic saline solution containing substantially 50 micrograms of an antigenic polysaccharide with a molecular weight of 60,000–5,000,000 whose main constituent is

N-acetyl, O-acetyl mannosamine phosphate, and which has substantially the following chemical composition expressed in terms of weight per 100 milligrams of sample:

mannosamine phosphate—H_2O, 67.24 milligrams
acetyl, 13.48 milligrams
sodium, 5.66 milligrams
water, 4.00 milligrams
calcium, less than 0.15 milligram
total amino acids, less than 1.00 milligram
nitrogen, 3.77 milligrams
phosphorous, 8.23 milligrams.

7. A meningococcal meningitis vaccine comprising an isotonic saline solution containing substantially 50 micrograms of an antigenic polysaccharide with a molecular weight of 60,000–5,000,000 whose main constituent is N-acetyl, O-acetyl neuraminic acid, and which has substantially the following chemical composition expressed in terms of weight per 100 milligrams of sample:

N-acetyl neuraminic acid—H_2O, 76.24 milligrams
O-acetyl, 5.80 milligrams
sodium, 2.51 milligrams
water, 9.50 milligrams
calcium, less than 0.15 milligram
total amino acids, less than 1.00 milligram
nitrogen, 3.81 milligrams
phosphorous, 0.0 milligram.

References Cited

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