PREPARATION OF THE TYPE-SPECIFIC POLYSACCHARIDE OF THE TYPE I MENINGOCOCCUS AND A STUDY OF ITS EFFECTIVENESS AS AN ANTIGEN IN HUMAN BEINGS*

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The demonstration by Francis and Tillett (1) and others (2–5) that the type-specific polysaccharides of pneumococci are antigenic in man and induce the production of antibodies in humans which protect mice against subsequent injection of virulent organisms, has led to numerous attempts to use these purified polysaccharides in active immunization (4, 6). Since the antipolysaccharide in Type I antimeningococcal sera has also been found to be effective in protecting mice, the capacity of several preparations of Type I meningococcal polysaccharide to induce precipitin formation in human volunteers was studied. Use was made of the colorimetric micro method for the estimation of antibody developed by Heidelberger and MacPherson (7) and applied in a study of the antibody response accompanying convalescence from pneumonia (8).

Preparations of the specific polysaccharide of the Type I meningococcus have been made and characterized by Scherp and Rake (9). Their materials, however, were shown to contain at least 20 per cent of a second substance which reacted with antimeningococcal sera (10) but was found not to remove protective antibody (11). A sample of polysaccharide (preparation 18 (9)) was generously supplied by Dr. H. W. Scherp for use in this investigation. In addition, since both glacial acetic acid and barium hydroxide had been used in purifying their polysaccharide, several lots were prepared in which the use of these fairly drastic reagents was avoided. The chemical and immunological properties of these materials were studied and it was found that, unlike preparation 18, they showed only a slight zone in the quantitative precipitin reaction where both antigen and antibody could be detected in the supernatant. No evidence of precipitin formation in humans was obtained with preparation 18, but in a few instances a small but significant antibody response to injection of

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several of the other polysaccharides was obtained, accompanied in three instances by the presence of protective antibody which was specifically removed by absorption with the same polysaccharide sample used in immunization.

Methods and Materials

Preparation of Polysaccharide—Freshly isolated lyophilized strains of Type I meningococci obtained from Drs. H. E. Alexander and J. J. Phair were transferred in peptone blood broth and then into Difco tryptose phosphate broth or to the casein hydrolysate medium described by Mueller and Hinton (12) but prepared without agar. The organisms were grown in 2-liter flasks, each containing 1 liter of medium, and incubated for 10 days at 37°. After the 1st day a pellicle was noted at the surface of the broth, and the flasks were shaken daily. The cultures were killed by addition of 95 per cent phenol to a concentration of 0.5 per cent. Contaminants were detected by examination of smears and by plating before addition of phenol and any contaminated flasks were discarded. The cell debris was removed in a Sharples centrifuge and when casein hydrolysate medium was used, the starch was digested with saliva. The broth was then concentrated to about one-tenth its original volume by ultrafiltration through alundum candles coated by dipping into a 4 per cent or 6.5 per cent parlodion solution in glacial acetic acid and then washed free of acid. It was found that small amounts of serologically active material passed through the 4 per cent membrane, but only traces could be detected after filtration through a 6.5 per cent membrane or through cellophane (*cf.* 9). In some instances the concentrated broth was then shaken with chloroform to remove protein (13), but it was found that complete removal of protein could not be effected.

In the case of lot M5, the concentrated tryptose broth (original volume, 8 liters) was shaken with chloroform, centrifuged, and 2 volumes of ethyl alcohol were added to the aqueous phase. The precipitate was dissolved in dilute sodium acetate and an equal volume of alcohol added. After several reprecipitations in this manner, a product (M5A) was obtained which was serologically inactive and was discarded. To the supernatant containing 2 volumes of alcohol 2 more volumes were added to precipitate the polysaccharide (M5B). M5B was redissolved in 80 ml. sodium acetate solution and precipitated with 2 volumes of alcohol. This precipitate was centrifuged, dissolved, reprecipitated with 1 volume of alcohol, the precipitate discarded, and the polysaccharide fraction M5B then precipitated by addition of alcohol to 4 volumes. 3 additional volumes of alcohol were added to the supernatants from the M5A reprecipitations and the precipitates were combined with M5B. The combined precipitates were dissolved in 64 ml. water and an equal volume of saturated ammonium sulfate was added. The precipitate was centrifuged and the supernatant dialyzed overnight against running water in a cellophane sac. Sodium acetate was added and the solution fractionally precipitated with alcohol into three fractions, M5B1, M5B2, M5B3, by successive addition of 2, 3, and 4 volumes of alcohol respectively. Each of these fractions was reprecipitated several times with alcohol, dissolved in water, and additional protein precipitated by addition of 2 volumes of saturated ammonium sulfate. After centrifuging, each supernatant was dialyzed free from ammonia, sodium acetate added, and the polysaccharide fraction precipitated with redistilled alcohol, washed with alcohol, and dried, yielding 247, 106, and 17 mg. of each fraction respectively.

With M6, 5 liters of tryptose broth were concentrated by ultrafiltration, shaken with chloroform, and the fraction precipitable by 1 volume of alcohol was discarded. The supernatant was divided into fractions M6B and M6C by addition of 2 and 4 volumes of alcohol respectively. Each of these fractions was dissolved and the protein precipitated by addition of 2 volumes of saturated ammonium sulfate and removed. The supernatants were dialyzed free from ammonia and precipitated with alcohol. M6B and M6C were recombined and dissolved in water and the solution was saturated with ammonium sulfate. The precipitate was centri-

fuged off, the supernatants dialyzed free from NH_4^+, and the polysaccharide (M6C) precipitated with alcohol. At this point its specific rotation was but +10° instead of +40° to 50° indicating considerable impurity and it was dissolved in about 5 ml. of water and a saturated solution of safranin (9) added until no more precipitation occurred. The precipitate was centrifuged off and washed with saturated safranin solution and with alcohol. It was then repeatedly dissolved in 20 per cent sodium acetate solution and precipitated with alcohol until free from safranin. The final precipitation was carried out with redistilled alcohol, the gummy precipitate washed several times with redistilled alcohol, and dried. Yield 48 mg.

M8 was made from 22 liters of tryptose broth. After concentration by ultrafiltration and shaking with chloroform, the material precipitable by 1 volume of alcohol was discarded. M8B was obtained by precipitation with 2 volumes of alcohol and M8C by precipitation between 2 and 3 volumes. M8B was reprecipitated twice with alcohol from sodium acetate solution, dissolved in about 5 ml. water, and 5 ml. solution containing 100 mg. protamine added. The white precipitate was centrifuged off, dissolved in 20 per cent sodium acetate solution, and reprecipitated with alcohol several times. It was finally precipitated with redistilled alcohol and dried. M8B protamine-precipitate, yield 235 mg. M8C was saturated with ammonium sulfate, the precipitate centrifuged off, and the supernatant dialyzed free from ammonia. Sodium acetate was added and the material precipitated with alcohol. The precipitate (266 mg.) was dissolved in water dialyzed against saline phosphate pH 7.4 and examined in the Tiselius electrophoresis apparatus (14), It showed two components and the fast component was separated in as high a yield as possible. This polysaccharide component (M8CF) was precipitated with alcohol and dried as described above—yield 59 mg. The unseparated M8C from the middle of the U tube was precipitated with protamine as described for M8B—yield 12 mg.

17 liters of casein hydrolysate broth (M9) were concentrated to about 1 liter by evaporation in large cellophane sacs in a stream of air from an electric fan and the polysaccharide precipitated by addition of 4 volumes of alcohol. The precipitate was dissolved and shaken with chloroform, the chloroform layer centrifuged off, and the material precipitable by 1 volume of alcohol removed and discarded. 3 more volumes of alcohol was added to the supernatant. The precipitate was dissolved in dilute sodium acetate and separated into two fractions M9B1 and M9B2 precipitable between 0–2 and 2–4 volumes of alcohol respectively. M9B2 was dissolved in water and the solution saturated with ammonium sulfate; the precipitate was removed and the supernatant dialyzed free from ammonia. Sodium acetate was added and the polysaccharide thrown down with alcohol. It was dissolved in 20 per cent sodium acetate and 4 volumes of methyl alcohol were added, the precipitate removed, and 4 volumes of ethyl alcohol added to the supernatant. The ethyl alcohol insoluble fraction was centrifuged, dissolved in 20 per cent sodium acetate, reprecipitated several times with alcohol, finally with redistilled alcohol, and dried. Yield 40 mg.

Some of the properties of the various Type I polysaccharide fractions prepared as above are given in Table I, together with data for sample 18 prepared by Scherp and Rake (9). The electrophoretic mobilities and relative concentrations of the components present are also included. The optical rotation, phosphorus content, and electrophoretic patterns of the M5 fractions indicate that some of the polysaccharide is precipitated by 2 volumes of alcohol but that most of it comes down between 2 and 4 volumes with less impurity. Fractional alcohol precipitation and ammonium sulfate precipitation at best yield products containing about 50 per cent polysaccharide. However, by treatment with safranin, separation by electrophoresis, or by removal of impurities by methyl

alcohol, products with chemical properties approaching those of sample 18 were obtained. Two of these, M6C and M8CF, showed but a single component in electrophoresis as did preparation 18, and the mobility found for all three samples was the same. The analytical data for M9B2 were closest to those of No. 18. The ash and phosphorus values on the two samples of protamine-precipitated polysaccharide as compared with those of M6C, M8CF, and M9B2 indicate a polysaccharide content about 55 to 60 per cent.

TABLE I

Chemical and Physical Properties of Type I Meningococcal Polysaccharide

Preparation No.	Ash as Na	N*	P*	$[\alpha]_D^*$	Electrophoretic pattern: Mobility at pH 7.4	Electrophoretic pattern: Amount of component present
	per cent	*per cent*	*per cent*	*degrees*	$\mu \times 10^{-5}$	*per cent*
M5B1	2.5	5.2	1.4	+19.4	−1.5 −10.6	88 12
M5B2		4.7	5.4	+23.6	−1.5 −11.6	52 48
M5B3		6.7	6.5	+26.6		
M6C	8.9	4.3	7.1	+41.5	−10.9	100
M8CF	10.8	5.6	7.3	+37	−10.2	100
M9B2	11.3	5.8	9.2	+51		
18 (Scherp)‡	9.74	4.20	8.93	+57	−11.3	100
M8C protamine precipitate	5.8	10.2	5.9	+8		
M8B protamine precipitate	5.4	12.1	5.7	+9		

* Calculated to the ash-free basis, except where no ash determinations are given.

‡ Taken from (9).

Reactions of Type I Polysaccharides with Homologous Antimeningococcal Horse Serum

Solutions containing 0.02 to 0.10 mg. of polysaccharide were added in duplicate at 0°C. to 1 ml. portions of Type I antimeningococcal horse serum H1095, obtained from Dr. Jules Freund of the New York City Department of Health Laboratories. The solutions were mixed and allowed to stand 48 hours in the icebox, centrifuged in the cold, and washed twice with

chilled saline. The washed precipitates were dissolved with a few drops of N/10 NaOH and analyzed for nitrogen by the micro Kjeldahl method as described by Heidelberger and Kendall (15). The supernatants were tested for the presence of excess antibody or polysaccharide by addition of polysaccharide or antiserum. (For a summary of quantitative immunochemical methods see reference 16.) The results are given in Table II.

M6C, M8CF, M8B protamine precipitate and M9B2 all reacted similarly with antiserum, precipitating a maximum of about 0.34 mg. antibody N from 1 ml. of serum. Sample 18, however, precipitated considerably more antibody N from 1 ml. of serum and its supernatants showed a broad range in which both antigen and antibody could be detected. The other preparations behaved

TABLE II

Antibody N Precipitated from 1 ml. Type I Horse Antiserum 1095 by Varying Amounts of Type I Meningococcal Polysaccharides

Polysaccharide added	M6C			M8CF			M8B Protamine precipitated			M9B2			18 (Scherp)		
	Antibody N precipitated	Supernatant + M6C	Supernatant + Antibody	Antibody N precipitated	Supernatant + M8CF	Supernatant + Antibody	Antibody N precipitated*	Supernatant + M8B protamine precipitated	Supernatant + Antibody	Antibody N precipitated	Supernatant + M9B2	Supernatant + Antibody	Antibody N precipitated	Supernatant + 18	Supernatant + Antibody
mg.	*mg.*			*mg.*			*mg.*			*mg.*			*mg.*		
0.02	0.248	+++	–	0.256	++	–	0.194	++++	–	0.262	+(+)	+(+)	0.318	+++	+
0.04	0.300	±(+)	++	0.298	++	++	0.251	++	–	0.286	–(+)	++++	0.406	++±	++
0.06	0.332	–(–)	+++	0.340	+	++++	0.329	±(±)	++	0.312	–(–)	++++	0.484	+++	+++
0.08	0.340	–(–)	++++	0.350	–(–)	++++	0.340	–(–)	+++	0.294	–(–)	++++	0.534	+++	+++
0.10	0.342	–(–)	++++	0.350	–(–)	++++	0.337	–	++++	0.278	–(–)	++++	0.594	+++	+++

Readings in parentheses after centrifugation.

* Corrected for additional protamine N in precipitate.

much more like a single substance, since their supernatants showed only a very narrow zone in which both components were present (7, 16). As would be expected from its protamine content equivalent weights of M8B protamine precipitate were less effective in precipitating antibody N from the horse serum, but the same amount of antibody was removed when excess polysaccharide was added. M9B2 appears to be most effective in precipitating antibody, but the maximum amount precipitated was somewhat low. M5B2 and M5B3 precipitated more than 0.90 mg. of antibody N from 1 ml. of H1095 indicating the presence of other immunologically reactive materials.

Immunization Studies with Type I Polysaccharide

The ability of a number of the polysaccharides to produce precipitins was tested in groups of human volunteers.

Immediately following an initial bleeding (designated by a zero subscript) two injections of polysaccharide in saline containing 0.01 per cent merthiolate were given intracutaneously or subcutaneously to each individual at 1 day intervals. Blood samples were drawn 3, 6, and 12 to 14 weeks following the injections. Antibody response was measured using the quantitative micro precipitin method of Heidelberger and MacPherson (7), described in detail in (8) in which the complement is first removed by the specific precipitate formed when 0.04 mg. of egg albumin (Ea) N is added to each of several 4.5 ml. portions of the human serum sample containing 0.4 mg. anti egg albumin N. After standing 48 hours in the icebox, 0.01 to 0.02 mg. of Type I meningococcal polysaccharide is added to aliquot portions of the supernatant from the Ea-anti Ea precipitate. The tubes were allowed to stand in the icebox for 1 week and were centrifuged in a refrigerated centrifuge and washed three times with chilled saline. The precipitates were dissolved with 1 drop of N/10 NaOH and analyzed for nitrogen by a modification of the Folin-Ciocalteau tyrosine method using as a standard a calibration curve

TABLE III

Antibody Formation in Individuals Injected with Type I Meningococcal Polysaccharide

Subject No.	Polysaccharide used	Amount injected	Antibody N per 4 ml. serum*			
			Before injection	After injection		
				3 wks.	6 wks.	12 to 14 wks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*
28	M5B3	0.05	0.001	0.009	0.009	0.013
43	M6C	0.10	0.002	0.006	0.009	0.011‡
47	M8CF	0.20	0.004		0.012	0.012
51§	M8CF	0.20	0.007	0.008	0.008	

* The same preparation used for immunization was used for antibody N determinations.

‡ 3, 6, and 12 weeks after two additional injections of 0.12 mg. M6C, values of 0.015, 0.012, and 0.012 mg. antibody N per 4 ml. serum were obtained.

§ Previously injected with 0.10 mg. of another crude preparation and showed a slight antibody response (0.007 mg. Ab N per 4 ml. serum) which was maintained.

obtained with known amounts of human gamma globulin N. Results are expressed in milligrams antibody N per 4 ml. serum.

A group of 22 volunteers received injections of 0.01 and 0.04 mg. of polysaccharide No. 18 (Scherp). Half of these were injected subcutaneously, the rest intracutaneously. No significant amounts of precipitin N were found in serum drawn 3, 6, and 12 to 14 weeks after injection. Eleven of these individuals received a second course of two injections of 0.05 and 0.20 mg. of polysaccharide and serum drawn at similar intervals also contained no measurable amounts of antibody N when tested with samples 18 or M5B3.

Nineteen volunteers were given injections of 0.01 and 0.04 mg. of crude polysaccharide M5B3. Only one of these 19 showed a significant antibody response 3 weeks later which was maintained for 12 weeks (No. 28, Table III). Four of the other individuals showed amounts of precipitin N of 0.008 to 0.009

mg. antibody N per 4 ml. serum in but a single serum sample and all other individuals showed no measurable antibody.

A third group of 14 individuals was given two injections each of 0.10 mg. M8CF. Half of these received the material as a protamine precipitate. In addition 3 persons received two injections of 0.02 and 0.08 mg. of M6C and 2 volunteers received two injections of 0.08 mg. M6C. Only 2 of the individuals injected with either M8CF or M6C showed a definite and persistent antibody response (No. 47, 43, Table III).

In three of the four instances in which definite precipitin formation resulted (Table III), mouse protection tests carried out by Dr. C. P. Miller's laboratory at the University of Chicago showed the presence of mouse protective antibodies. These antibodies were no longer present in the serum after removal of the antipolysaccharide. No protective antibodies were found in three samples of serum from an individual injected with preparation 18 (Scherp) who did not produce precipitins. A 1:30 dilution of serum 43, drawn 12 weeks after the second series of injections, protected $\frac{3}{4}$ of the inoculated mice against 1000 M.L.D. of Type I meningococci, as did a 1:30 dilution of serum 47 drawn 12 weeks after injection. A 1:1.4 dilution of the 12 week sample of serum 51 protected $\frac{3}{4}$ of the mice against 1000 M.L.D. After absorption of the Type I antibody with M5B3 or with M6C and M8CF, no protective power remained in the supernatant.

DISCUSSION

The purification of Type I meningococcal polysaccharide from autolyzed broth by methods which avoid the use of strong acid or alkali is attended with considerable difficulty. Although small yields of relatively pure material have been obtained by fractional precipitation with alcohol and removal of protein with ammonium sulfate, followed by precipitation with safranin, separation of impurities by electrophoresis or by methyl alcohol, as yet none of these methods has proved adequate when maximum recovery of polysaccharide was desired. This is perhaps due to the tendency of the polysaccharide to combine with protein and other substances present because of its high phosphorus content (*i.e.* acidic groups) and to carry down other impurities in the course of the usual precipitations.

The data in Table I show that the properties of the various polysaccharides are in general similar to those reported by Scherp and Rake (9) in showing a high ash and phosphorous content and 4 to 5 per cent N. The Scherp and Rake sample, No. 18, and M6C and M8CF showed but a single component in electrophoresis with a mobility of about 11 at pH 7.4, indicating a large number of acidic groups on the polysaccharide. Since preparation 18 was found by Scherp to contain 20 per cent of a serologically reactive impurity (10), which may have affected the analytical data, the true optical rotation, N, P, and ash contents

of pure polysaccharide may differ somewhat from the given values. This might in part account for the variation in the analytical constants of the different polysaccharides. The formation of an insoluble protamine-polysaccharide compound which is soluble in sodium acetate may prove to be of considerable value in further purification studies. For many purposes the protamine-polysaccharide complex may even prove to be as satisfactory as the polysaccharide itself.

The quantitative precipitin data obtained in Table II indicate that M6C, M8CF, M9B2 as well as the protamine-polysaccharide complex contain much smaller amounts of immunologically reactive impurities than does sample 18, since their supernatants showed but a very slight zone in which both antigen and antibody were present, whereas the 18 supernatants gave tests for both antigen and antibody over a very wide range (*cf.* 10, 16, 17). M6C, M8CF, M9B2, and M8B protamine precipitate all removed about the same maximum amount of antibody nitrogen from the serum. Equal amounts of the protamine-polysaccharide complex nitrogen were somewhat less effective in removing antibody as would be expected from their lower polysaccharide content. From the immunological standpoint, therefore, it appears that these polysaccharide preparations are much closer than preparation 18 to being a single substance.

The results of the immunization studies in human beings indicate that the Type I meningococcal polysaccharide is a very poor antigen compared with the pneumococcal type-specific polysaccharides. No antibodies were obtained in 22 individuals inoculated with preparation 18 and only four definite responses resulted among 38 individuals immunized with M5B3, M6C, or M8CF. Two of these four were immunized with the purified preparations M6C and M8CF. In the three instances tested, the production of precipitins after immunization was associated with the presence of Type I antibodies capable of protecting mice. These antibodies were specifically removed by absorption with polysaccharide. Whether these positive results indicate that the polysaccharide is weakly antigenic cannot be definitely established. The recent demonstration (11) that Type I meningococci contain another type-specific antigen which is also associated with mouse protection, makes it conceivable that in these few instances antibody to this other antigen was formed. It is quite possible that traces of this antigen could be present even in the purified polysaccharides

SUMMARY

1. Methods for preparing small amounts of Type I meningococcal polysaccharide which are electrophoretically homogeneous and contain only traces of other immunologically reactive material are given.

2. These polysaccharides are very poor antigens in man but in a small number of instances definite amounts of precipitin and protective antibody were formed.

3. The significance of these findings is discussed.

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