IMMUNOLOGICAL STUDIES ON A NEW PREPARATION OF TYPE SPECIFIC POLYSACCHARIDE FROM PNEUMOCOCCUS TYPE I

By BACON F. CHOW, Ph.D.

(From the Department of Biochemistry, Peiping Union Medical College, Peiping, China)

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The isolation of polysaccharide from the three most common types of pneumococci by Avery and Heidelberger (1–3) has made it possible to explain the biological type specificity on a chemical basis and is a definite advance in the study of the antigenic structure of the bacteria. Since the isolation both European (4) and American (5–7) laboratories have also obtained polysaccharides from Type I Pneumococcus, which are apparently different from Avery and Heidelberger's soluble specific substance. Thus several names have been given by various authors to the polysaccharides of Type I Pneumococcus. Fortunately, the general confusion has been greatly clarified by the recent paper of Avery and Goebel (8). They isolated from the same bacteria a polysaccharide in a more complete form, through the omission of an alkali treatment in their preparation. This acetyl polysaccharide, as they called it, has labile acetyl groups attached to the soluble specific substance. The antigenicity in mice depends on the presence of the acetyl group.

In this report, we shall show that an even more complete polysaccharide can be isolated from the autolyzed broth culture of Type I Pneumococcus by slightly modifying Avery and Goebel's method.

1. Preparation of the Polysaccharide of Pneumococcus Type I from the Autolyzed Broth Culture

Culture.—The Pneumococcus Type I strain used for this work was obtained from The Rockefeller Institute, New York. Its virulence was such that 10^{-8} cc. of an 18 hour broth culture killed mice within 48 hours.

Composition of Culture Medium.—Virulent Type I pneumococci were grown in meat infusion broth of pH 7.6 containing 0.5 per cent NaCl and 1.0 per cent dextrose.

30 liters of the broth culture was seeded with Pneumococcus Type I. After

12 hours of incubation at 37°C., 10 cc. of a 10 per cent sterile glucose solution was added to each liter of broth in order to increase the growth of bacteria. After 7 or 8 days, most of the bacteria had autolyzed. The pH of the autolyzed broth was 5.2. The culture was centrifuged to get rid of the bacterial debris and the unautolyzed cells. The slightly opalescent supernatant was concentrated to 3 liters *in vacuo*, the temperature of the culture being maintained below 37°C. 4 liters of alcohol (95 per cent) was added to the concentrated broth culture. After standing for 30 hours, the supernatant liquid was siphoned off and the precipitate was packed by centrifuging in four 250 cc. bottles. The precipitate in each bottle was washed with 50 cc. of water and recentrifuged. The washing was continued until the supernatant gave only a faint Molisch test. The combined supernatants were concentrated *in vacuo* to 600 cc. and cooled with ice. 70 cc. of 50 per cent trichloroacetic acid was added, and the suspension was centrifuged. The precipitate was washed 2 or 3 times with cold 10 per cent trichloroacetic acid. The combined supernatants and washings were again concentrated *in vacuo* to 200 cc. 10 volumes of acetone were added to precipitate the polysaccharide. After standing overnight the clear supernatant was siphoned off and the precipitate was collected by centrifugation. The polysaccharide was repeatedly washed with 20 cc. portions of water and a small amount of insoluble protein residue was discarded. The combined washings were again reprecipitated with 10 volumes of acetone. The polysaccharide was again washed with water and precipitated with acetone. This process was repeated 4 or 5 times. Finally, the clear polysaccharide solution was rapidly dialyzed in the cold against 0.001 N HCl. When the dialysis was complete, the polysaccharide was precipitated with 10 volumes of acetone, centrifuged, and dried with acetone and ether. The yield was 1.1 gm. The product was white. In 1 per cent solution it gave strong positive Molisch, but negative biuret, Millon's, or xanthoproteic tests.

2. Immunological Properties of the Newly Prepared Polysaccharide

It was found that when Type I antipneumococcus rabbit serum was absorbed with the acetyl polysaccharide, the absorbed serum still effectively protected mice from an otherwise fatal dose of Type I pneumococci. This fact means that either there is more than one kind of protective antibody in the rabbit antiserum or that the acetyl polysaccharide is still not in the natural and most complete form, or possibly both. The following experiments will show that our polysaccharide is still more complete than the acetyl polysaccharide, for it possesses the immunological properties of the latter, and it will also precipitate an antipneumococcus Type I rabbit serum absorbed with the acetyl polysaccharide.

Precipitin Reaction of the New Polysaccharide in Antipneumococcus Rabbit and Horse Sera, Absorbed and Unabsorbed by Acetyl Polysaccharide

It was shown elsewhere (9) that Type I antipneumococcus rabbit serum absorbed with the acetyl polysaccharide would still react with our polysaccharide and that our polysaccharide precipitated about 3 times as much of antibody from the immune rabbit serum as the acetyl polysaccharide.

It was therefore of interest to ascertain whether Type I antipneumococcus immune horse serum previously absorbed with the acetyl polysaccharide would react with the new polysaccharide. Our results

TABLE I

Determination of the Total Antibody Content of an Immune Rabbit Serum by the Quantitative Agglutination Method

Experiment No.	Volume of serum	Total volume of heat-killed vaccine	Total protein in the precipitate	Protein in total vaccine	Agglutinin protein per cc. of serum
	cc.	*cc.*	*mg.*	*mg.*	*mg.*
A	1.00	6.00	14.6	2.10	12.5
B	1.00	8.00	13.7	1.44	12.3

confirmed the findings of Avery and Goebel (8) in that unlike the immune rabbit serum, the corresponding horse immune serum previously absorbed with the acetyl polysaccharide would neither react with our polysaccharide nor agglutinate Type I pneumococci.

This difference in the homologous types of immune rabbit and horse sera is not unexpected, for it is now well known that immune sera from these two species differ both chemically and immunologically (10–12).

Per Cent of the Total Antibody Protein in Immune Rabbit Serum Precipitable by Our Polysaccharide

It was of interest to find what portion of the antibody in the immune serum was precipitated by the acetyl and by our polysaccharides. To find the total antibody content of the immune serum, we have followed the method of quantitative agglutination of Heidelberger and Kabat (13). The results are shown in Table I.

The procedure used is as follows: In Experiment A, 1 cc. of the immune serum was pipetted into each of two 15 cc. centrifuge tubes. 1 cc. of heat-killed vaccine prepared according to Heidelberger and Kabat's direction was added to each tube. The mixture was kept at 0°C. for 48 hours and then centrifuged at 0°C. The precipitate was washed with 2 cc. of cold saline and to the supernatant was added another 1 cc. of the vaccine. The mixture was again kept at 0°C. for 48 hours, and centrifuged as before. The process was repeated until there was no appreciable agglutination 48 hours after the addition of vaccine. The precipitates were combined and analyzed for nitrogen.

In Experiment B, 1 cc. of the immune serum was used, but the vaccine was diluted twofold. Otherwise the procedure was exactly the same. It will be noticed from Table I that it only took 8 cc. of the diluted vaccine to carry down the same amount of agglutinin as 6 cc. of the undiluted. The results in the table are the average of duplication determinations.

TABLE II

Protective Action of Antipneumococcus Rabbit Serum (Type I) before and after the Absorption with the Newly Prepared Polysaccharide

Serum dilution	Unabsorbed		Absorbed with the polysaccharide	
1:400	S	S	S	S
1:200	S	S	S	S
1:80	S	S	S	S
1:20	—	—	S	S
1:10	—	—	S	S

S = survival for at least 5 days after the inoculation of culture.

Control mice receiving no serum died 48 hours after the injection of 10^{-8}, 10^{-7}, 10^{-6} cc. of 18 hour broth culture.

The last column in Table I shows that there are 12.4 mg. of antibody protein in each cc. of the immune serum. From the same serum our polysaccharide precipitated some 4.35 mg. protein or 35 per cent of the total antibody, whereas the acetyl polysaccharide precipitated 1.34 mg. protein or 11 per cent.

Although the newly prepared polysaccharide is more complete, yet it does not remove all the protective antibodies from the immune serum. Table II shows that immune serum previously absorbed by our polysaccharide still afforded effective protection in mice against 500,000 M.L.D. of virulent Type I Pneumococcus. All mice received an intraperitoneal injection of 0.5 cc. of various dilutions of sera together with 0.5 cc. of 1:200 dilution of 18 hour broth culture of Pneumococcus Type I.

In Table III, it may be seen that neither the acetyl nor our polysaccharides, removed all the antibody from immune rabbit serum.

Though the absorbed serum could still agglutinate Type I pneumococci, the agglutination titre of the serum absorbed with our polysaccharide was greatly reduced.

Antigenicity of the Polysaccharide in Mice and Rats

An important guide of the immunological properties of the polysaccharide from Type I Pneumococcus is its antigenic response in mice. Many investigators have definitely shown that their polysaccharides derived from Type I Pneumococcus lost their antigenicity when boiled with alkali. Avery and Goebel have shown that the loss of antigenicity is due to the loss of acetyl groups of the polysaccharide.

TABLE III

Agglutination of Type I Pneumococcus in Immune Rabbit Serum before and after Absorption with the Polysaccharides

Serum	Final dilution of sera							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Unabsorbed	++ +	++++	++++	++++	++++	+++	++	±
Absorbed with the acetyl polysaccharide	++++	++++	++±	++	+	−	−	−
Absorbed with the newly prepared polysaccharide	++	++	+	±	−	−	−	−

It was therefore of interest to see whether our polysaccharide, which is apparently more complete, would also lose its antigenicity in mice.

0.5 cc. of 1:million dilution of the polysaccharide was injected intraperitoneally into each of a group of six mice. Another group of six mice received identical amounts of the same polysaccharide previously heated at 100°C. in 0.05 N NaOH for 30 minutes. Three doses were given to each group at the intervals of 3 days. 3 days after the last injection, the mice were given intraperitoneally 0.5 cc. each of a virulent culture of Type I Pneumococcus. The results are given in Table IV.

It is of interest to note that our preparation is apparently more resistant to alkali than those from other laboratories. However, it must not be taken to mean that alkali will not destroy the antigenicity of our polysaccharide, for a prolonged boiling in 0.05 N will convert the polysaccharide to serologically nonreactive substances.

The antigenic action of our polysaccharide in rats was also observed. Two rats weighing about 300 gm. each received intravenous injections through the tail veins, 0.5 cc. of 1:million solution of the polysaccharide. Another pair received

TABLE IV

Antigenic Action in Mice of the Newly Prepared Polysaccharide before and after Alkali Treatment

Dilution of pneumococcus culture (Type I)	Normal mice		Immunized mice injected with the newly prepared polysaccharide		Immunized mice injected with the alkali-treated polysaccharide	
cc.						
10^{-4}	—	—	S	S	S	S
10^{-5}	D 30	D 48	S	S	S	S
10^{-6}	D 30	D 49	S	S	S	S
10^{-7}	D 30	D 49	—	—	—	—
10^{-8}	D 30	D 30	—	—	—	—

S = survived. D followed by a number = death occurred at the number of hours. —— = not done.

TABLE V

Protection of Mice against Fatal Infections with Type I Pneumococci by Rat Sera Immunized with the New Polysaccharide

Serum dilution	Source of serum					
	From rats receiving a total of 1 cc. of 1:million solution of the polysaccharide		From rats receiving a total of 1 cc. of 1:10,000 solution of the polysaccharide		From normal rats receiving no polysaccharide	
Undiluted	S	S	S	S	D 30	D 44
1:10	S	S	S	S	D 30	D 36
1:100	S	S	S	S	D 30	D 36

Virulence Control

Dilution of culture	Result	
cc.		
10^{-6}	D 30	D 44
10^{-7}	D 44	D 48
10^{-8}	D 44	D 44

S = survival. D followed by number = death occurred at the designated number of hours.

0.5 cc. of 1:10,000. The injections were repeated after 3 days. 3 days after the second injection the rats were bled to death and the sera from the two groups were collected. That these rat sera protected mice from a lethal dose of Type I Pneumococcus can be seen from Table V.

Antigenicity of the New Polysaccharide in Rabbits

None of the polysaccharides prepared in different laboratories has been reported to be antigenic in rabbits. It was therefore interesting to ascertain the antigenicity of our polysaccharide in rabbits. For that purpose, six rabbits were given intravenously three daily injections of 1 cc. of the polysaccharide (1:1000 solution), followed by a rest period of 3 days. Three courses of injection were given in all, so that each rabbit received a total amount of 9 mg. After the third course of injections, the rabbits were bled from the heart and the sera were tested for the presence of type specific protective antibodies, precipitins, and agglutinins.

The results of the protective action in mice are given in Table VI. The results in Table VI show that the sera from the immunized rabbits protected mice against only 100 M.L.D. of Type I pneumococci. Although it must be admitted that they do not show conclusively that the new polysaccharide did stimulate antigenic response in rabbits, yet mice receiving sera from the immunized rabbits were more resistant to the fatal infection of Type I pneumococci. It was also found that rabbits immunized with one-tenth or one-hundredth of the above amount, *i.e.* 0.9 mg. or 0.09 mg. respectively, gave sera which showed no protective action.

Precipitin and agglutination reactions were also performed with the immunized rabbit sera, but no observable reaction could be detected in either instance. This is not surprising since the protection test showed the presence of only a very small amount of protective antibody.

Treatment with Acids and Base.—The effect of hydrogen ion concentration on the precipitative activity and antigenicity of our polysaccharide in mice and rabbits was studied. When a 1:1000 solution of the new polysaccharide was heated for ½ hour at the boiling temperature of water in various acid solutions, such as 0.5 N acetic acid, 0.05 N HCl, 0.2 N HCl, the qualitative precipitin titre was not decreased. The product failed to react with the immune rabbit serum absorbed with the acetyl polysaccharide, but it still precipitated the immune rabbit serum absorbed with the deacetylated polysaccharide of Avery and Heidelberger.

The results are also shown in Table VII. This fact together with the observation that the antigenicity of the polysaccharide is not lost by the acid treatment (see Table VIII) suggests that the acetylated polysaccharide may be the hydrolytic product of our polysaccharide. The chemical nature of the hydrolyzed group will be studied later.

TABLE VI

Protection of Mice against Fatal Infection with Type I Pneumococci, by Rabbit Sera Immunized with the New Polysaccharide

Type I culture	Sera from rabbits												Virulence control	
	1		2		3		4		5		6			
cc.														
10^{-4}	D 44	D 44	D 40	D 30	D 44	D 48	D 40	D 48	D 48	D 48	D 50	D 48	—	—
10^{-5}	S	D 72	S	S	S	D 76	D 80	D 76	D 72	S	D 72	D 80	—	—
10^{-6}	S	S	S	S	S	S	S	S	S	S	S	S	D 44	D 40
10^{-7}	—	—	—	—	—	—	—	—	—	—	—	—	D 40	D 42
10^{-8}	—	—	—	—	—	—	—	—	—	—	—	—	D 40	D 30

Mice weighing 15 to 20 gm. were used. Each mouse received 0.2 cc. serum and 0.5 cc. culture. Sera from these six rabbits before immunization showed no protection in mice against a fatal dose of Pneumococcus Type I.

All mice used for the experiment reported in Table VIII received three doses of 0.5 cc. of 1:million dilution of the original, or acid-treated polysaccharide. The immunizing procedure was identical with that previously given.

TABLE VII

Precipitin Reaction of the Polysaccharide after the Treatment with 0.500 N Acetic Acid

Serum	Final dilution of the polysaccharide hydrolyzed by acids				
	1:10,000	1:40,000	1:160,000	1:640,000	1:2,560,000
Unabsorbed	++++	++++	++++	++	+
Absorbed with the acetyl polysaccharide	−	−	−	−	−
Absorbed with the deacetylated polysaccharide	+++±	++	+	±	−

TABLE VIII

Antigenic Action in Mice of the Newly Prepared Polysaccharide before and after Acid Treatments

Dilution of pneumococcus culture (Type I)	Normal mice		Mice immunized with the newly prepared polysaccharide		Mice immunized with the acid-treated polysaccharide	
cc.						
10^{-4}	—	—	S	S	S	S
10^{-5}	D 30	D 48	S	S	S	S
10^{-6}	D 30	D 48	S	S	S	S
10^{-7}	D 30	D 49	—	—	—	—
10^{-8}	D 30	D 30	—	—	—	—

S = survived. D followed by a number = death occurred at the number of hours. — — = not done.

TABLE IX

Precipitin Reaction of the Polysaccharide after the Treatment with 0.05 N NaOH

Serum	Final dilution of the polysaccharide hydrolyzed by alkali				
	1:10,000	1:40,000	1:160,000	1:640,000	1:2,560,000
Unabsorbed	++++	++++	+++	++	+
Absorbed with acetyl polysaccharide	−	−	−	−	−
Absorbed with deacetylated polysaccharide	+++±	++	+	±	−

The effect of the hydroxyl ion was also studied. As shown before, the antigenicity was not destroyed when the polysaccharide was heated at 100°C for 30 minutes in 0.05 N NaOH solution. The

TABLE X

Protection of Mice against Fatal Infection with Type I Pneumococci, by Rabbit Sera Immunized with the New Polysaccharide and with Its Acid Hydrolytic Product

Type I culture	Sera from rabbits immunized with												Virulence control	
	The original new polysaccharide						The acid hydrolytic product							
cc.														
10^{-5}	D 48	S	D 80	D 60	D 48	D 60	D 44	D 44	D 30	D 44	D 64	D 44	—	—
10^{-6}	S	S	S	S	S	S	D 60	D 44	D 44	D 44	D 72	D 60	D 30	D 44
10^{-7}	—	—	—	—	—	—	—	—	—	—	—	—	D 30	D 44
10^{-8}	—	—	—	—	—	—	—	—	—	—	—	—	D 44	D 44

precipitative activity with the immune rabbit serum absorbed with the acetyl polysaccharide was destroyed. However, it could still react with the unabsorbed immune serum and with serum absorbed with the deacetylated polysaccharide. The results are recorded in Table IX.

The effect of acid on the antigenicity of our polysaccharide in rabbits was also studied. It was found that the polysaccharide obtained after the acid treatment was no longer antigenic, for the sera from three rabbits immunized with the acid-treated polysaccharide afforded no protection on mice against a virulent strain of Type I pneumococci. The result of the protective action of the immunized rabbit sera is given in Table X.

SUMMARY

A type specific polysaccharide has been isolated from the autolyzed broth of Type I Pneumococcus by a modified Avery and Goebel's method.

The newly prepared polysaccharide reacts with the homologous immune rabbit serum which has been completely absorbed with the acetyl polysaccharide of Avery and Goebel.

The newly prepared polysaccharide produces passive immunity in mice and rats and possibly in rabbits.

The antigenicity is not lost on boiling in acid or alkaline medium, but the precipitative activity is decreased.

In conclusion, it has been shown that the polysaccharide from Type I Pneumococcus, as isolated by a slight modification of Avery and Goebel's method, is a more complete antigen.

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