THE IMMUNOLOGICAL PROPERTIES OF THE HETEROPHILE ANTIGEN AND SOMATIC POLYSACCHARIDE OF PNEUMOCOCCUS

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The isolation and properties of the heterophile antigen of pneumococcus have been described in a previous communication (1). This substance, obtained from the cellular debris of an autolyzed culture of a rough variant of Type I pneumococcus[1] is a lipocarbohydrate constituted from molecules of an acetylated amino sugar, a second hexose, phosphoric acid, and a high molecular weight fatty acid. The heterophile antigen, or F carbohydrate, of pneumococcus is closely related both chemically and immunologically to the somatic or C carbohydrate obtained from autolysates of the same microorganism (2). The heterophile antigen is believed to have a polysaccharide moiety identical with the C carbohydrate to which is bound in firm chemical union a fatty acid of high molecular weight. The latter is thought to endow the heterophile antigen with properties which distinguish it from the somatic carbohydrate.

When pneumococci are permitted to autolyze the somatic polysaccharide passes into solution, whereas the heterophile antigen remains firmly associated with the bacterial detritus. The F polysaccharide can be separated from the cellular debris by appropriate chemical procedure and has been isolated as an amorphous, non-dialyzable substance, soluble in water, and gives faintly opalescent solutions which foam on shaking. The heterophile antigen has been isolated free from protein, polypeptides, and nucleic acid, and is homogeneous when examined by electrophoresis. On chemical analysis the C and F carbohydrates of pneumococcus are scarcely distinguishable, yet certain points of difference, both chemical and immunological, have been demonstrated. For example, the F carbohydrate yields some 6 per cent of a fatty acid on hydrolysis, whereas the C substance does not. Furthermore, the two carbohydrates have different mobilities when examined by electrophoresis. The following account shows that these chemical distinctions are reflected in immunological differences which further distinguish the substances from each other.

[1] This strain will be referred to as the IR pneumococcus, and the antiserum prepared by immunization of rabbits with these organisms will be referred to as anti R serum. The heterophile antigen or lipocarbohydrate of the IR pneumococcus will be referred to as the F polysaccharide, whereas the somatic carbohydrate is termed the C polysaccharide.

EXPERIMENTAL

Immunization of Rabbits with Heat-Killed Pneumococci.—The bacteria from a 12 hour broth culture of a rough variant of Type I pneumococcus (hereafter referred to as the IR pneumococcus) were collected by centrifugation, suspended in one-tenth the original volume of saline, and heated at 100° for 5 minutes. Rabbits were immunized by the intravenous injection of 1 cc. of the bacterial suspension for 6 consecutive days. The animals were bled 5 days after the last injection, and the sera tested for sheep cell hemloysins and precipitins for the somatic C carbohydrate. One to two courses of immunization sufficed to produce potent antisera.

Determination of Hemolysins.—½ cc. of varying dilutions of serum was mixed with ½ cc. of a 1:10 dilution of fresh guinea pig complement, and one drop of 50 per cent washed sheep cells added. After 30 minutes' incubation at 37° the tubes were centrifuged, examined, and the highest dilution of serum giving complete hemolysis was noted. Twice the reciprocal of the highest serum dilution giving complete hemolysis is the number of units of hemolysin per cc. of serum. In all instances preliminary test bleedings were made on the rabbits before immunization and the natural hemolysin determined. Rabbits showing more than ten units per cc. of serum were discarded.

Precipitin Tests.—½ cc. of serial dilutions of the pneumococcus C polysaccharide in saline was added to 0.2 cc. of serum plus 0.3 cc. of saline. After incubation for 2 hours at 37° and refrigeration overnight the precipitin reactions were recorded.

Results of Immunization

The production of sheep cell hemolysins did not parallel the appearance of C precipitins in the rabbits immunized as above. There was great individual variation in response to immunization in all groups of rabbits as can be seen in Table I. Some animals, after one course of immunization, developed as much as 800 units of hemolysin per cc. Other showed less than 100 units. In general these observations agree with those of Bailey and Shorb (3), who noted that prolonged immunization resulted in a decrease in hemolytic titer. In no case were we able to procure sera containing more than 1600 hemolytic units per cc., and in most instances a serum containing 400 units per cc. was considered very good. From some animals potent hemolytic sera were obtained in which the titer for the C polysaccharide was relatively weak. From others excellent precipitating antisera were procured containing a low hemolytic titer. Some sera were obtained possessing both high hemolytic titer and potent C precipitating antibodies.

Immunization with Bacterial Detritus

Bailey and Shorb (3) report that the heterophile antigen of pneumococcus is not destroyed by autolysis and the results of the experimental work described below agree with their observations.

Suspensions of living pneumococci (rough variant Type I) were allowed to autolyze for 72 hours at 37°. The bacterial detritus, containing no Gram-positive cells, was centrifuged, washed, and used as an immunizing antigen. The supernatant liquid from the centrifuged autolysate, containing C polysaccharide, nucleoprotein, pig-

TABLE I

Hemolysins and Preciptins in Sera of Rabbits Immunized with the Intact Cells of I R Strain of Pneumococcus

Rabbit No.	First course		Second course		Third course		Fourth course	
	Hemolytic units	C precipitin	Hemolytic units	C precipitin	Hemolytic units	C precipitin	Hemolytic units	C precipitin
1	200	++	<100	+++±	100	++±	200	++++
2	0	+++	0	++++	<100	++	0	+
3	200	++++	200	++++	100	++±	400	++++
4	<100	++	0	+++±	100	+++	200	++++
5	400	++	200	+++	200	+++	200	+++
6	100	+	100	+++±				
9	800	+++	800	++++				
10	400	+±	400	++				
11	100	++	400	+++±				
12	400	++±	400	+++				
19	0	0	0	0	100	±		
20	<100	±	0	++++	0	+++		
21	50	0	50	+	0	+++±		
23	<100	±	*		*			
24	200	+	0	+	0	±		
27	100	0	400	+±	100	+		
9-19	100	+	1600	++++	*			
9-20	600	+++±	200	+++	160	+++	100	+++
9-22	100	++	400	++	80	+	<100	+
9-23	200	+	400	+++	80	+++	<100	+

++++ indicates heavy disk-like precipitate (final concentration of C polysaccharide 1:50,000).

0 indicates no precipitate.

* Died.

TABLE II

Hemolysins and C Precipitins in Sera of Rabbits Immunized with Autolytic Products of I R Pneumococcus

Rabbit No.	Immunized with	First course		Second course	
		Hemolytic units	C* precipitin	Hemolytic units	C precipitin
49-04	Supernatant liquid from autolyzed pneumococci	0	0	0	0
49-05	" "	0	0	0	0
49-06	" "	0	0	0	0
49-07	Pneumococcus detritus	200	+	100	0
49-08	" "	400	+	200	+
49-09	" "	200	+	400	+

\+ indicates slight flocculent precipitate.

* Final concentration of C polysaccharide used for testing 1:50,000.

ment, and nucleic acid was filtered through a Berkefeld candle to remove any particles of debris and the filtrate likewise used as an immunizing antigen.

In Table II it can be seen that the bacterial detritus served as an excellent antigen for the production of hemolytic antisera, whereas the filtrate failed to stimulate the production of hemolytic antibodies. It can also be noted that the hemolytic sera resulting from injections of detritus contained little or no precipitins for the C polysaccharide.

Immunization with C and F Polysaccharides

Rabbits were injected intravenously with solutions of C and F polysaccharides containing 2 mg. of carbohydrate per cc. The rabbits received 1 cc. of carbohydrate solution daily for 6 days. 5 days after the last injection the animals were bled and

TABLE III

Hemolysins in Sera of Rabbits Immunized with C and F Polysaccharides

Rabbit No.	Immunized with	First course Hemolytic units	Second course Hemolytic units
13	F polysaccharide	200	800
14	" "	50	400
15	" "	50	200
28	C polysaccharide	0	0
30	" "	0	0
31	" "	0	0

the sera tested for the presence of hemolytic antibodies and C precipitins. Two courses of injections were given.

From the results presented in Tables III and IV it can be seen that the F polysaccharide functions as an excellent antigen. The production of potent hemolytic antibodies is accompanied by the formation of precipitins for the homologous F antigen and to some extent for the C polysaccharide as well. The latter, on the other hand, fails to function antigenically, for neither hemolysins nor precipitins are produced when rabbits are injected with the carbohydrate. Prolonged immunization of rabbits with a solution of C polysaccharide likewise failed to stimulate the production of antibodies. A total of 84 mg. was given to a group of animals over a period of 7 weeks. At no time during the course of immunization was it possible to demonstrate either hemolysins or precipitins in the sera of these rabbits.

From the above experiments it can be concluded that intact pneumococci, the insoluble detritus from pneumococcus autolysates, and the purified F polysaccharide all stimulate in rabbits the formation of sheep cell hemolysins.

As a rule the appearance of C carbohydrate precipitins occurs during immunization with intact pneumococci, whereas little or none appear when the bacterial detritus or F polysaccharide is used as the immunizing agent. The C polysaccharide, on the other hand, though closely related chemically to the F carbohydrate, is completely inert antigenically.

TABLE IV

Precipitins in Sera of Rabbits Injected with C and F Polysaccharide

Rabbit No.	Injected with	Test antigen*	
		F polysaccharide	C polysaccharide
13	F polysaccharide	++	+
14		+	tr.
15		±	tr.
16		++	±
28	C polysaccharide	—	0
29		—	0
30		—	0
31		—	0

++ indicates flocculent precipitate.
tr. indicates trace reaction.
0 indicates no precipitation.
* Final concentration of test antigen 1:50,000.

TABLE V

Inhibition of Hemolysins in Anti R Rabbit Serum by C and F Polysaccharides

Inhibiting substance	Dilution of inhibiting substance											
	1:500	1:1000	1:2000	1:4000	1:8000	1:16,000	1:32,000	1:64,000	1:128,000	1:256,000	1:512,000	1:1,024,000
F polysaccharide	0	0	0	0	0	0	0	0	0	0	0	++
C polysaccharide	±	+	+±	+±	++	++±	+++	++++	++++	++++	++++	++++

0 indicates no hemolysis.
++++ indicates complete hemolysis.

Inhibition of Hemolysis

In a previous publication differences in the chemical properties of the C and F polysaccharides were described (1). That these differences are reflected in differences in the immunological properties of the two carbohydrates can be

demonstrated in a number of ways. In the preceding section it was shown that sheep cell hemolysins are produced by the immunization of rabbits with solutions of the F polysaccharide, but that such antibodies were not produced when the C carbohydrate was used as the immunizing agent. A second striking difference between the two polysaccharides can be demonstrated by means of the hemolysis inhibition test (4). Varying amounts of the substance to be tested are added to a constant amount (2 units) of hemolytic antiserum. After incubation for 30 minutes sheep cells and complement (2 units) are added and the degree of inhibition of hemolysis noted after 30 minutes' further incubation. The results presented in Table V demonstrate that 2 micrograms of F polysaccharide inhibit the lysis of sheep cells by pneumococcus heterophile antiserum. On the other hand a thousand times this amount of C polysaccharide is necessary to produce complete inhibition.

Agglutination of Pneumococci

In order to determine whether the antibody responsible for the agglutination of the rough variant of Type I pneumococcus by homologous antisera is identical with the C or F precipitating antibodies the following experiment was performed:—

Portions of anti R rabbit serum were absorbed respectively with the C and F polysaccharides. Complete removal of the corresponding precipitins and sheep cell hemolysins resulted. The absorbed sera were compared with the original unabsorbed serum in respect to their ability to agglutinate the IR pneumococcus.

The bacterial cells from a 12 hour broth culture of an R strain derived from Pneumococcus Type I were washed, then resuspended in saline, and killed by the addition of merthiolate in a concentration of 1:10,000. Cell suspensions prepared in this way are sterile and the microorganisms remain Gram-positive for several days at room temperature. Moreover, under these conditions the bacteria do not clump spontaneously as do heat-killed bacteria. ½ cc. of bacterial vaccine is added to ½ cc. of serial dilutions of sera, and the mixture incubated 2 hours at 37° and refrigerated overnight.

The results, recorded in Table VI, indicate that the complete removal of precipitins for the C and F polysaccharides and of sheep cell hemolysins does not alter the agglutinin titer of the serum.

In Table VII it can be seen that the sera of rabbits immunized with pneumococcus detritus and which contain C and F precipitins and hemolysins, agglutinate the intact organisms. Sera obtained by immunization with the purified F polysaccharide, although containing precipitins for this substance, cause little or no agglutination of the organisms. From these observations it is apparent that in addition to antibodies which precipitate the C and F carbohydrates and those which cause the lysis of sheep cells, antisera to the IR

pneumococcus contain independent agglutinating antibodies (*cf.* Heidelberger and Kendall (5) on the behavior of anti C on absorption by R pneumococci).

Toxicity of the F Polysaccharide

Lipocarbohydrates derived from Gram-negative bacilli are known to be toxic. Thus Morgan (6) has shown that 0.1 mg. of the lipocarbohydrate from

TABLE VI

Agglutinins in Anti R Rabbit Serum before and after Absorption with C and F Polysaccharides

Anti R pneumococcal rabbit serum	Final dilution of serum						
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400
1. Unabsorbed	++++	++++	++++	++++	++	+	0
2. Absorbed with F polysaccharide	++++	++++	++++	++++	++	+	0
3. Absorbed with C polysaccharide	++++	++++	++++	++++	++	+	0

++++ indicates complete flocculation with clear supernatant.
0 indicates no agglutination.

TABLE VII

Agglutination of I R Pneumococcus in Sera of Rabbits Immunized with Autolyzed Detritus and F Polysaccharide

Rabbit No.	Immunized with	Final dilution of serum						
		1:10	1:20	1:40	1:80	1:160	1:320	1:640
49-08	Pneumococcus detritus	+++	+++	++±	++	+	0	0
49-09	" "	+++	+++	+++	++±	++	+	0
13	F polysaccharide	++	++	+	0	0	0	0
16	" "	++	+	0	0	0	0	0
P*	I R pneumococcus	+++	+++	+++	+++	+++	++	0
	Normal rabbit serum	++	+	0	0	0	0	0

+++ indicates complete agglutination.
0 indicates complete agglutination.

Shiga bacillus is sufficient to kill mice. More recently the lipocarbohydrate of typhoid bacillus has likewise been found to have toxic properties (7). In order to determine whether the F polysaccharide of pneumococcus is toxic, a series of twelve rabbits were injected with serial dilution of the pneumococcus lipocarbohydrate, using as the highest dose 20 mg. of polysaccharide dissolved in 2 cc. of saline. The animals were observed over a period of 2 weeks, none showed toxic symptoms and all animals survived. Mice injected intraperi-

toneally with identical doses likewise failed to develop toxemia, and none succumbed.

Quantitative Precipitin Titrations

It has been observed that potent anti R rabbit sera precipitate both the C and F polysaccharides at concentrations of 1:2,000,000. Complete absorption of such sera with the F polysaccharide removes not only the precipitins for the

TABLE VIII

Quantitative Precipitin Titration of Anti R Rabbit Serum

Experiment No.	Antigen added (x)	Nitrogen in precipitate	Ratio of antibody N to antigen in precipitate (R)*	Supernatants tested with			Hemolytic units in supernatant
				C polysaccharide	F polysaccharide	Anti R serum	
Unabsorbed serum and C polysaccharide							
	mg.	*mg.*					
1	0.01	0.088	8.8	++++	++++	0	200
2	0.03	0.199	6.6	+++±	+++	0	75
3	0.06	0.376	6.3	+++	++	0	50
4	0.08	0.448	5.6	++	+±	0	25
5	0.10	0.530	5.3	+	+	±	12
6	0.15	0.534	—	tr.	tr.	+	0
7	0.20	0.542	—	tr.	tr.	+±	0
Unabsorbed serum and F polysaccharide							
8	0.025	0.105	4.2	++++	+++	tr.	125
9	0.050	—	—	+++	+++	tr.	65
10	0.075	0.288	3.8	+++	++	tr.	40
11	0.10	0.343	3.4	+++	+±	tr.	16
12	0.15	0.361	2.4	++	+	±	8
13	0.15	0.413	2.7				
14	0.20	0.410	2.05	++	±	±	0
15	0.30	0.429	—	+	±	+	0
16	0.50	0.505	—	±	±	+	0
17	1.00	0.573	—	±	0	+±	0

++++ indicates heavy disk-like precipitate.

0 indicates no precipitate.

* In calculating R it is assumed that all the antigen added is present in the precipitate. This is true up to the equivalence point. The nitrogen contents of the antigens used (6 per cent) is so small that no correction for nitrogen from this source was made in calculating R.

homologous substance but those for the C polysaccharide as well. On the other hand, sera absorbed with the C carbohydrate still possess precipitins for the F polysaccharide, but in considerably reduced titer.

In order to obtain more exact information concerning the immunological relationship of these two carbohydrates, a quantitative study of the precipitin

reaction titrations was made using an antiserum containing both hemolytic and precipitating antibodies. Since one of the properties of the F polysaccharide is its ability to neutralize the hemolysins of anti R rabbit serum, these antibodies were removed from a portion of the serum by absorption with boiled sheep stromata. The C and F precipitins in the absorbed and unabsorbed serum were then determined quantitatively and the results compared in order to ascertain whether the hemolytic antibodies play a part in the precipitin reaction.

The absorption was carried out by adding 15 cc. of a suspension of boiled sheep stromata to 30 cc. of pooled anti R serum. After standing at 0° for 24 hours the

TABLE IX

Quantitative Precipitin Titration of Anti R Rabbit Serum Absorbed with Boiled Sheep Stromata

Experiment No.	Antigen added (x)	Nitrogen in precipitate	Ratio of antibody nitrogen to antigen in precipitate (R)
Absorbed serum and C polysaccharide			
	mg.	*mg.*	
18	0.025	0.189	7.6
19	0.050	0.297	5.9
20	0.100	0.457	4.6
21	0.200	0.530	2.65
22	0.500	0.492	—
Absorbed serum and F polysaccharide			
23	0.025	0.075	3.0
24	0.050	0.138	2.8
25	0.100	0.239	2.3
26	0.200	0.271	1.35
27	0.500	0.368	—
28	1.000	0.423	—

stromata were removed by centrifugation and the serum was tested for sheep cell hemolysins. Since the latter were completely removed, the absorbed serum was diluted with saline to a concentration half that of the original serum.

Quantitative precipitin titrations were performed by the method of Heidelberger and Kendall (8). All procedures were carried out at 0°. Throughout the reaction range excess of antigen or antibody was determined in the supernatant liquid after centrifugation of the immune precipitate. The precipitated antibody nitrogen was determined by the micro Kjeldahl method.

The results of the quantitative titrations on the pooled anti R rabbit serum both before and after absorption with boiled sheep stromata are summarized in Tables VIII and IX and Figs. 1 and 2. From the findings it is evident that the C and F polysaccharides differ in their capacity to precipitate the

antibodies in anti R rabbit serum. A given weight of C polysaccharide precipitates much more antibody nitrogen than does the same amount of F

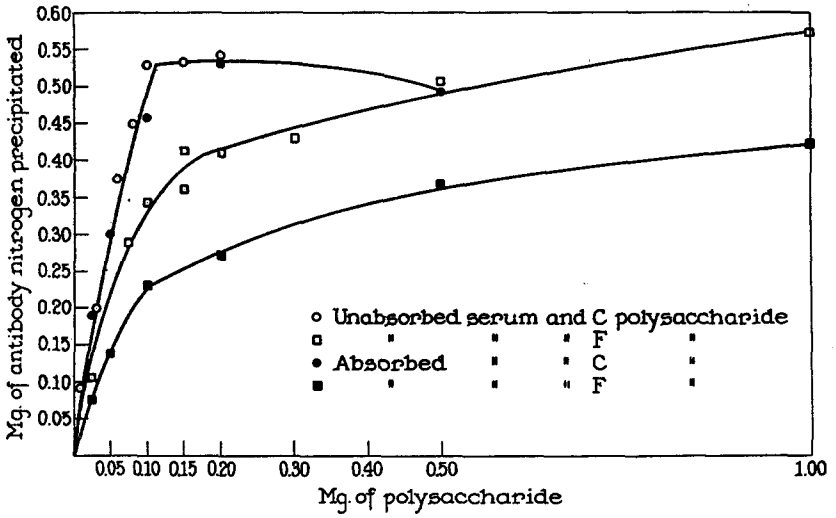

FIG. 1

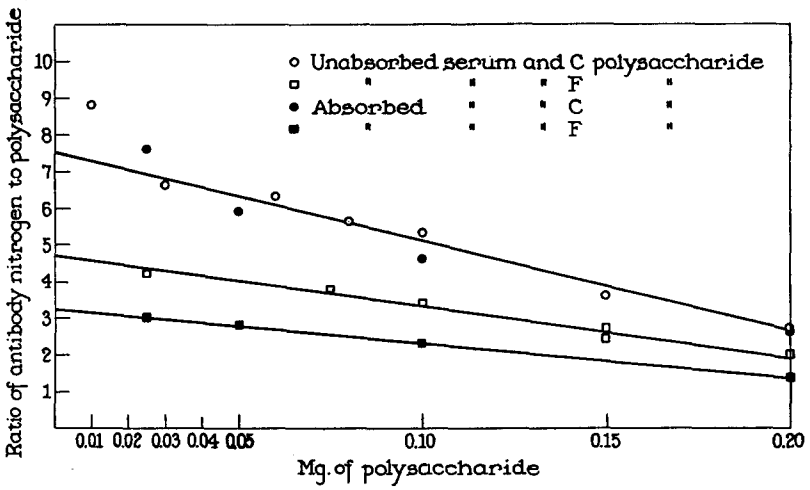

FIG. 2

polysaccharide. Moreover, the shapes of the two curves differ, indicating that the F polysaccharide is distinct and different from the C polysaccharide, and is not merely the latter contaminated with inert material.

This distinction is still further emphasized when one compares the reaction of C and F polysaccharides in the stromata-absorbed serum. It can be seen that absorption of the serum with stromata does not alter the amount of antibody precipitable by the C polysaccharide. However, the quantity of antibody precipitable by the F polysaccharide has been reduced by one-third on absorption with boiled sheep stromata.

TABLE X

Summary of the Results of the Quantitative Precipitin Titration in Terms of the Heidelberger-Kendall Equation

Serum	Antigen	R	A (calculated)	A (observed)	Antibody protein precipitated per cc. of serum at point R
			mg.	*mg.*	*mg.*
Unabsorbed serum.....	C polysaccharide	3.75	0.56	0.53	1.77
Absorbed serum.....	" "	3.75	0.56	—	1.77
Unabsorbed serum.....	F polysaccharide	2.35	0.41	0.413	1.29
Absorbed serum.....	" "	1.65	0.275	—	0.87

R = ratio of antibody N to antigen at the equivalence point.

A = antibody nitrogen precipitated at point R.

The values in column 6 are obtained by multiplying the values for A by the protein conversion factor 6.3 followed by a division by 2, since the constants R and A were calculated for 2 cc. of serum used.

The relationship between the C and F polysaccharides as precipitinogens can be best analyzed by applying the equation derived by Heidelberger and Kendall.

$$r = 2R - \frac{R^2x}{A}$$

where: r is the ratio of antibody nitrogen/antigen in the precipitate,

R is a constant which usually approximates the ratio of antibody nitrogen/antigen in the precipitate at the point of first antigen excess.

A is a constant which approximates the amount of antibody nitrogen in the precipitate at point R.

x is the amount of antigen in the precipitate at point r. The constants A and R are characteristic of the particular antigen-antibody system under investigation.

In Fig. 2 the values for r from Tables VIII and IX are plotted against the corresponding values for x. From this figure the constants R and A of the equation are determined for each quantitative titration. The constants together with the calculated antibody protein precipitated at point R are recorded

in Table X. It is evident from Fig. 2 that the quantitative relationship between the experimental values for r and x is linear, and hence the precipitin reaction of the C and F polysaccharides in anti R rabbit serum is typical of precipitin reactions in general.

DISCUSSION

From the data presented above it is evident that anti R rabbit serum contains a complex mixture of antibodies including hemolysins for sheep red blood cells, agglutinins for the intact bacterial cells as well as precipitins for the C and F carbohydrates. A study of the quantitative precipitin reactions of the C and F polysaccharides in the antiserum both before and after removal of the hemolytic antibody with sheep stromata reveals certain interesting relationships. From the data presented in Table X it is seen that 1.77 mg. of antibody protein per cc. of serum is precipitable by the C polysaccharide both in the unabsorbed and absorbed serum. The same quantities of F polysaccharide, on the other hand, precipitate considerably less antibody protein in the unabsorbed serum and still less in the serum which has been absorbed with sheep cell stromata. The curves given in Fig. 2 and the equation constants presented in Table X further emphasize the marked difference between the reaction of the two polysaccharides in the antiserum.

The reaction of the C polysaccharide in the antiserum is typical of precipitin reactions between polysaccharide haptens and their homologous antibodies. The precipitin reaction follows the equation of Heidelberger and Kendall (8) through the equivalence point. The addition of excess polysaccharide does not result in the precipitation of additional antibody.

In contrast the reaction of F polysaccharide in the antiserum is not typical of an homologous system. The precipitin reaction in this case consists of two phases, the first of which, up to the equivalence point, is typical of precipitin reactions obeying the equation of Heidelberger and Kendall. In the second phase of the precipitin reaction, where excess antigen is always present in the supernatant liquid, the amount of antibody precipitated is nearly proportional to the antigen added. This reaction between F polysaccharide and stromata-absorbed serum bears a striking analogy to the cross reaction between the Type VIII capsular polysaccharide and Type III antipneumococcus serum studied by Heidelberger and coworkers (9) and lends support to the view that the C and F polysaccharides are closely related chemically but are serologically distinct.

During the course of our study on the quantitative precipitin titration it was observed that when sufficient C polysaccharide had been added to the serum to reach the equivalence point, the supernatant, after removal of the immune precipitate, was devoid of hemolytic antibodies. Since there is no excess of C polysaccharide in the supernatant at the equivalence point, one can conclude

that the hemolysin has been removed and constitutes a part of the immune precipitate. It is interesting to note that the quantity of C polysaccharide necessary to neutralize the hemolysins as determined by the inhibition test is far in excess of that required to remove them by precipitation.

If one absorbs the serum with sheep cell stromata to remove the hemolysins and then determines the amount of antibody precipitable by the C polysaccharide it is observed that the antibody precipitated is identical with that precipitated in the unabsorbed serum by all concentrations of antigen up to and even beyond the equivalence point. From this one can conclude that the hemolytic antibody represents so small an amount of the total antibody protein that it cannot be determined under the conditions of our experimental procedure. This observation is in direct agreement with that of Brunius (10) who found that the purified horse kidney heterophile antibody equivalent to 200 hemolytic units weighed only 0.01 mg.

A study of the quantitative precipitation of the F polysaccharide in the anti R rabbit serum likewise reveals that the amount of carbohydrate required to precipitate the hemolysins is considerably less than that needed to neutralize these same antibodies as determined by the inhibition test. The C and F polysaccharides are very nearly equivalent in their capacities as precipitating antigens for the removal of the hemolytic antibody, although as an inhibitor of the latter, the F polysaccharide is a thousand times more potent than is the C carbohydrate.

In addition to sheep cell hemolysins the unabsorbed serum contains an antibody which is absorbed by boiled sheep stromata, precipitated by F polysaccharide, but not precipitated by C polysaccharide. The existence of this type of antibody must be assumed to account for the presence of more F precipitable protein in the unabsorbed serum than in the stroma-absorbed serum. This antibody is not precipitable by C polysaccharide since the amount of C precipitable protein in the unabsorbed serum is identical with that in the stromata-absorbed serum. From column 4 of Table X it is evident that this antibody amounts to 0.42 mg. of protein per cc. of serum.

The existence of this type of antibody is also borne out by qualitative absorption experiments. Whole serum which has been completely absorbed with C polysaccharide still precipitates with F polysaccharide at an antigen concentration of 1:250,000. Since this antibody protein reacts with both F polysaccharide and boiled sheep stromata but not with C carbohydrate the specificity of this antibody is probably dependent upon the lipid component of the F polysaccharide.

In conclusion it should be pointed out that the unabsorbed serum contains an agglutinin for R pneumococci which is immunologically unrelated to either the C or F polysaccharides. Sera from which all precipitins for these polysaccharides have been removed still agglutinate R pneumococci in undiminished

titer. The serum prepared by immunization with the detritus from autolyzed R cells contains agglutinins in high titer. However, a serum of equal hemolytic and precipitin titer obtained by immunization with the purified F polysaccharide is devoid of the power to agglutinate pneumococci.

Whether the C polysaccharide occurs in the intact pneumococcus as such or in chemical combination with some cellular constituent which renders it antigenic, or whether it is an artifact derived from the F polysaccharide during the autolytic process are questions which cannot be answered with complete assurance. One can conceive of a cellular constituent, a C-lipid complex, which could give rise to precipitins for the intact antigen as well as for the carbohydrate haptene portion of the molecule dissociated from its lipid constituent. The lipocarbohydrate of the Shiga bacillus is apparently a substance of this sort (6). There are, however, certain experimental facts which indicate that the C carbohydrate antibodies in the sera of rabbits immunized with intact pneumococcal cells are separate and distinct from those evoked by the C-lipid complex (F polysaccharide). Rabbits immunized with the latter give rise to antibodies which are weak in precipitins for the C haptene but which precipitate the homologous antigen strongly.

Immunization of rabbits with intact heat-killed Gram-positive pneumococci, on the other hand, gives rise to potent precipitating antisera for the C haptene. Furthermore, a study of the quantitative precipitin reaction in these antisera reveals that the precipitation of the C carbohydrate follows the course of an homologous reaction, whereas that of the F polysaccharide resembles heterologous precipitin reactions. Since the purified C carbohydrate is not by itself antigenic, this would indicate that in the intact pneumococcus it is combined in some way to form an antigenic complex distinct in its specificity from that of the antigenic F polysaccharide.

CONCLUSIONS

1. The lipocarbohydrate or F polysaccharide derived from a rough variant of Type I pneumococcus (I R) is antigenic in rabbits and gives rise to precipitins and sheep cell hemolysins. The somatic or C carbohydrate on the other hand is not antigenic.

2. Antisera for the rough variant of Type I pneumococcus contain also bacterial agglutinins immunologically unrelated to the C and F precipitins and the heterophile antibody.

3. A study has been made of the quantitative precipitin reaction of the C and F polysaccharides in the serum of rabbits immunized with the I R pneumococcus clarifying certain of these relationships.

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