THE RELATION BETWEEN THE TYPE SPECIFIC CARBOHYDRATES OF PNEUMOCOCCI AND THE BLOOD GROUP SPECIFIC SUBSTANCE A

By ERNST WITEBSKY, M.D., ERWIN NETER, M.D., AND HARRY SOBOTKA, PH.D.

(From the Laboratories of The Mount Sinai Hospital, New York)

(Received for publication, February 9, 1935)

The correlation between Forssman's antigen and the blood group specific substance of human blood group A is well established (1). The content of either one of these antigens in a number of bacteria has been studied by several authors. Forssman's antigen was found in various strains of *B. shigae*, *B. paratyphosus*, *B. lepisepticus*, etc. It also occurs in pneumococci (2, 3). The relationship between pneumococci and the blood group specific substances A and B has been the subject of recent investigations by Baily and Shorb (4). These authors observed agglutination of human blood cells by various antipneumococcic sera irrespective of type. Concerning the carbohydrate nature of the blood group substance A, we refer to Landsteiner (5), and Brahn, Schiff, and Weinmann (6). Freudenberg and Eichel (7) succeeded in isolating from urine of men belonging to group A a carbohydrate which in its chemical structure is closely related to the type specific carbohydrate of pneumococci prepared by Avery, Heidelberger, and Goebel.

The following study deals with the relationship between the soluble specific substances of pneumococci and the blood group specific substance A. In a study of this kind, one has to take into account the widespread occurrence in animals of Forssman's antigen and blood group antigens as well as of the corresponding antibodies.

Methods

There are various methods by which to demonstrate the presence of blood group specific substances in cells, tissues, organs, and juices; first, the specific inhibition of the iso-agglutination of human blood cells; second, the complement

fixation and the hemolysis inhibition with group specific antisera, especially of group A; finally, the active immunization which results in the formation of specific antibodies. Although the rabbit is most suitable for this purpose, not all rabbits produce group specific A-antibodies, a high percentage of these animals containing the A-antigen in their organs, and are hence unable presumably to produce group A-antibodies. On the other hand, rabbits lacking the A-antigen (they usually possess normal A-agglutinins) actually produce A-antibodies.

The so called hemolysis inhibition test seems to be especially useful for the elucidation of the relationship between the type specific carbohydrates of pneumococci and the blood group specific substance of men. This method is based on the following principle (11). Group specific A-antisera often contain a high titer of sheep cell hemolysins. Material containing group A substance combines with the group specific antibodies of a group A-antiserum, including the minor antibody which causes sheep cell hemolysis. If sheep cells are added afterwards, hemolysis does not occur.

Action on Sheep Cells of Blood Group Specific A-Antiserum, Treated with Type Specific Pneumococcus Carbohydrate

Experiment 1.—Decreasing amounts of a 1 per cent solution of the soluble specific substances of Pneumococcus Types I, II, and III, and of a 0.5 per cent solution of peptone, known to contain group A substance, were made up to 0.2 cc. with physiological saline, mixed with 0.2 cc. of a suitable dilution of a group specific A-antiserum, and allowed to stand for 20 minutes at room temperature. Then 0.2 cc. of a 5 per cent sheep cell suspension and 0.2 cc. guinea pig serum in a dilution of 1:10 (complement) were added. Hemolysis, as observed after 30 minutes, is recorded in Table I.

As will be seen, soluble specific substance, Type III, markedly inhibits the sheep cell hemolysis, caused by the group specific A-antiserum, while the soluble specific substance, Type I, has no effect; polysaccharide, Type II, exerts a moderate inhibitory influence. In this and in every similar experiment, a sheep cell antiserum was used as control to prove the group specific character of the inhibition of the sheep cell hemolysis by the group specific A-antiserum.

Other polysaccharides, for example dextrin, gum arabic, gum ghatti, gum acacia, and a specific carbohydrate isolated from tubercle bacilli (B C G) by Dr. E. Chargaff proved negative.

Comparison of Acetylated and Acetyl-Free Soluble Specific Substance, Type I, in Hemolysis Inhibition Test

Experiment 2.—The ineffectiveness of the Type I polysaccharide prompted us to compare its action with that of the acetyl carbohy-

TABLE I

Hemolysis of Sheep Cells by Complement and A Antiserum Mixed with Soluble Specific Pneumococcus Carbohydrate (Hemolysis Inhibition Test)

Amount of 1 per cent solution of SSS (or peptone) in 0.4 cc. of mixture with antiserum	Degree of sheep cell hemolysis by mixture of A antiserum with			
	SSS I	SSS II	SSS III	Peptone
cc.				
0.2	c.	0	0	0
0.1	c.	tr.	0	0
0.05	c.	c.	0	0
0.025	c.	c.	0	0
0.0125	c.	c.	tr.	0
0.0062	c.	c.	m.	tr.
0	c.	c.	c.	c.

c. = complete hemolysis.
ac. = almost complete hemolysis.
m. = moderate hemolysis.
tr. = traces of hemolysis.
0 = no hemolysis.

TABLE II

Hemolysis of Sheep Cells by Complement and A Antiserum Treated with Soluble Specific Pneumococcus Substances

Amount of 1 per cent solution of SSS in 0.4 cc. mixture with antiserum	Degree of hemolysis by mixture of A antiserum with		
	SSS I acetylated	SSS I de-acetylated	SSS III
cc.			
0.1	0	m.	0
0.05	0	ac.	0
0.025	0	c.	0
0.0125	0	c.	tr.
0.0062	0	c.	m.
0.0031	0	c.	ac.
0.0015	0	c.	c.
0.00078	0	c.	c.
0.00039	0	c.	c.
0.00019	tr.	c.	c.
0.000095	tr.	c.	c.
0	c.	c.	c.

For explanation of abbreviations see Table I.

drate, recently isolated by Avery and Goebel (8) as the genuine type specific antigen. The test was set up as follows:

Decreasing amounts of acetyl polysaccharide, of de-acetylated polysaccharide of Type I, and also of soluble specific substance, Type III, made up to 0.2 cc., were mixed with 0.2 cc. of a suitable dilution of a group specific A-antiserum (rabbit) and kept for 20 minutes at room temperature. To these were added 0.2 cc. of a 5 per cent suspension of sheep cells and 0.2 cc. guinea pig serum in a dilution of 1:20. Hemolysis was recorded after 60 minutes incubation at 37°C.

There is a striking difference between the acetyl and the de-acetylated polysaccharides, Type I. The acetyl polysaccharide inhibits sheep cell hemolysis by group specific A-antiserum to a high degree, while the de-acetylated product is almost completely ineffective. The inhibitory potency of the acetyl polysaccharide, Type I, towards the group specific sheep cell hemolysis exceeds that of the soluble specific substance, Type III.

Complement Fixation Test of Group Specific A-Antiserum and Pneumococcus Carbohydrates

Experiment 3.—The question arose whether similar results could be obtained with the complement fixation test. To this end, an experiment was set up in the following manner.

Decreasing amounts (total volume 0.2 cc.) of acetyl polysaccharide, Type I, of de-acetylated polysaccharide of the same type, of polysaccharide, Type III, and of peptone (Witte) were treated for 1 hour at 37°C. with 0.2 cc. of a group specific A-antiserum (dilution 1:10), and 0.2 cc. of guinea pig serum (1:20). To these were added 0.4 cc. of an equal mixture of 5 per cent sheep cell suspension and a suitably diluted sheep cell antiserum (rabbit). The resulting hemolysis was noted (α) after 15 minutes, and (β) after 30 minutes incubation at 37°C.

Table III shows that the acetyl carbohydrate caused a marked complement fixation with the group specific A-antiserum, while the de-acetylated derivative was completely negative in this respect. The soluble specific substance, Type III, showed only a slight positive reaction.

Inhibition of Iso-agglutination of Human Group A Corpuscles by Pneumococcus Polysaccharides

Experiment 4.—The inhibitory influence of the soluble specific substance of pneumococci upon the iso-agglutination of human red blood

TABLE III

Hemolysis of Sheep Cells by Sheep Cell Antiserum and Complement after Treatment of the Latter with A Antiserum and Soluble Specific Substances of Pneumococci (Complement Fixation Test)

Amount of 1 per cent solution of SSS (or peptone) in 0.6 cc. mixture with A antiserum and complement	Degree of hemolysis							
	SSS I acetylated		SSS I de-acetylated		SSS III		Peptone	
cc.	α	β	α	β	α	β	α	β
0.05	0	m.	c.	c.	0	c.	0	0
0.025	0	0	c.	c.	tr.	c.	0	m.
0.0125	0	0	c.	c.	c.	c.	0	c.
0.0062	0	0	c.	c.	c.	c.	0	c.
0.0031	0	0	c.	c.	c.	c.	tr.	c.
0.0015	0	tr.	c.	c.	c.	c.	m.	c.
0.00078	0	ac.	c.	c.	c.	c.	c.	c.
0.00039	0	c.	c.	c.	c.	c.	c.	c.
0	c.	c.	c.	c.	c.	c.	c.	c.

α = hemolysis after 15 minutes.
β = hemolysis after 30 minutes.
Other abbreviations as in preceding tables.

TABLE IV

Agglutination of Human Red Blood Cells of Group A by Serum Group O after Treatment of the Latter with Soluble Specific Substance

Amount of 1 per cent solution of SSS in 0.2 cc. mixture with human O serum	Iso-hemagglutination by human O serum treated with					
	SSS I acetylated		SSS I de-acetylated		SSS III	
cc.	α	β	α	β	α	β
0.05	−	−	++	++++	++	++++
0.0167	−	+	++	++++	++	++++
0.0056	−	++	++	++++	++	++++
0.0018	−	++	++	++++	++	++++
0.0006	±	+++	++	++++	++	++++
0	++	++++	++	++++	++	++++

− = no agglutination.
+ = slight agglutination.
++ = marked agglutination.
+++ = strong agglutination.
++++ = very strong agglutination.
α = after 1 hour.
β = after 10 hours.

cells by normal human serum containing the corresponding iso-agglutinins was demonstrated as follows:

Decreasing amounts (total volume 0.1 cc.) of acetyl and de-acetylated polysaccharide, Type I, and of polysaccharide, Type III, were incubated for 20 minutes at room temperature with 0.1 cc. of inactivated human serum belonging to group O (dilution 1:1). To this was added 0.1 cc. of a 1 per cent suspension of human blood cells belonging to group A. Agglutination was noted (α) after 1 hour, and (β) after 10 hours incubation at room temperature.

The acetyl polysaccharide, Type I, displayed a marked inhibitory influence on the group specific iso-agglutination of human blood cells of group A, in contrast to the de-acetylated compound of the same type. However, in comparison with the inhibition of sheep cell hemolysis by a group specific A-antiserum, much larger amounts of the acetyl polysaccharide were necessary. It may be remarked that the iso-agglutination of blood cells of group B was not influenced at all in a parallel experiment.

Schiff, Akune, and Weiler (9, 10) have described an agent in the feces and in the saliva which destroys the group specific substances. These authors regard the "blood group enzyme" as a product of the body itself, while Witebsky and Satoh (12, 13) and recently Sievers (14) are of the opinion that this agent augments itself and is not a secretion product of the organism. The question as to the nature of this blood group-destroying agent need not be discussed here. The one fact may be mentioned that one can obtain sterile Berkefeld filtrates of feces (12) which also destroy the blood group specific substances.

In view of the above described relationship between the soluble specific substances of pneumococci and the blood group substances of man, we investigated the effects of such a Berkefeld filtrate of feces on the soluble specific pneumococcus substances.

Effect of Blood Group Enzyme on Acetyl Polysaccharide, Type I, as Demonstrated by Hemolysis Inhibition Test

Experiment 5.—0.5 cc. of a ⅓ per cent solution of acetyl polysaccharide, Type I, was mixed with 0.5 cc. of an effective feces filtrate, and with 0.5 cc. of the same filtrate, inactivated by heating for 20 minutes at 60°C. These mixtures were kept at 37°C. overnight. Then, the hemolysis inhibition test was set up as

in Experiment 1. The effectiveness of feces filtrates was proved in this and similar experiments by its ability to destroy the blood group substances.

Table V shows that the acetyl polysaccharide lost its potency to inhibit sheep cell hemolysis by a group specific A-antiserum after being digested with effective feces filtrate, while it still inhibited the sheep cell hemolysis when digested with feces filtrate, inactivated by heat. The loss of reactivity toward the group specific A-antiserum of

TABLE V

Hemolysis of Sheep Cells with Complement and A Antiserum Treated with Acetylated SSS Type I Which Had Been Digested Previously with Feces Filtrate

Amount of 1/6 per cent solution of SSS in 0.4 cc. of mixture with antiserum	Degree of hemolysis in hemolysis inhibition test with acetylated SSS I digested with Feces filtrate			
	Effective		Ineffective	
cc.	α	β	α	β
0.2	tr.	c.	0	0
0.1	m.	c.	0	0
0.05	m.	c.	0	0
0.025	m.	c.	0	0
0.0125	ac.	c.	0	0
0.0062	ac.	c.	0	0
0.0031	ac.	c.	0	c.
0.0015	ac.	c.	0	c.
0.00078	ac.	c.	m.	c.
0.00039	c.	c.	m.	c.
0.00019	c.	c.	ac.	c.
0.00009	c.	c.	ac.	c.

Abbreviations as in Table I.
α = after 20 minutes.
β = after 40 minutes.

the acetyl carbohydrate by digestion with feces filtrate could also be demonstrated by the complement fixation test.

It may be mentioned in this connection, that the soluble specific substance of pneumococci, Type III, also loses its reactivity toward the group specific A-antiserum by digestion with effective feces filtrate.

Inhibition of Iso-agglutination by Acetyl Polysaccharide Abolished by Blood Group Enzyme

Experiment 6.—The following observation confirms the destructive influence of active feces filtrate on the acetyl carbohydrate, Type I.

Equal amounts of 0.5 cc. of 0.2 per cent solution of the acetyl substance were mixed with 0.5 cc. effective and 0.5 cc. inactivated feces filtrate, and kept at 37°C. overnight. Decreasing amounts of these mixtures, made up to 0.1 cc., were then digested for 20 minutes at room temperature with 0.1 cc. of an inactivated human serum belonging to blood group O (solution 1:1). After this, 0.1 cc. of a 1 per cent suspension of human blood cells, group A, was added. The resulting agglutination was recorded (α) after 20 minutes and (β) after 50 minutes incubation at room temperature.

As can be seen from Table VI, the acetyl polysaccharide, after having been digested with active feces filtrate, has lost its potency to

TABLE VI

Agglutination of Human Blood Cells of Group A by Serum of Group O Treated with Acetylated Type I Polysaccharide Which Had Been Digested Previously with Feces Filtrate

Amount of digested 1/10 per cent acetyl SSS I solution in 0.2 cc. mixture with group O serum	Degree of iso-hemagglutination by human O serum mixed with acetylated SSS Type I, digested with			
	Active Feces filtrate		Inactivated	
cc.	α	β	α	β
0.1	−	−	−	++
0.05	−	−	+	++++
0.025	−	−	+	++++
0.012	−	±	+	++++
0.006	−	+	++	++++
0	+++	++++	+++	++++

Symbols as in Table IV.
α = agglutination after 20 minutes.
β = agglutination after 50 minutes.

inhibit the agglutination of human blood cells of group A by a serum of group O as in Experiment 4.

Effect of Blood Group Enzyme on the Precipitability of Acetyl Substance by Pneumococcus Type I Antiserum, Previously Absorbed with De-acetylated Polysaccharide

Experiment 7.—A Pneumococcus Type I antiserum, previously absorbed with de-acetylated polysaccharide, still precipitates the acetyl substance according to Avery and Goebel (8). We mixed, therefore, 10 cc. of a 1:1 diluted Pneumococcus Type I antiserum (horse) with 0.5 cc. of a 0.1 per cent solution of the de-

acetylated compound and kept it for 2 hours at 37°C. and then overnight in the ice chest. The resulting heavy precipitate was centrifuged, and the entire procedure was repeated twice with the supernatant fluid. The final supernatant fluid was called "absorbed Type I antiserum." The test was set up as follows: Acetyl carbohydrate was digested with effective and with ineffective feces filtrate at 37°C. overnight. Then, these specimens were tested against the absorbed Type I antiserum simultaneously with acetyl polysaccharide and with de-acetylated polysaccharide as controls. Decreasing amounts of these four specimens (total volume 0.1 cc.) were mixed with 0.3 cc. of the absorbed Type I antiserum and

TABLE VII

Precipitation of Soluble Specific Substance of Type I Pneumococcus with Absorbed Pneumococcus Type I Antiserum

Amount of 1/10 per cent solution of soluble specific substances in 0.4 cc. total volume	Degree of precipitation of Pneumococcus Type I antiserum, previously treated with de-acetylated SSS Type I, when mixed with SSS Type I							
	Acetylated						De-acetylated	
	Digested with				Control			
	Active Feces filtrate		Inactivated Feces filtrate					
cc.	α	β	α	β	α	β	α	β
0.1	−	++	−	+	−	+	−	−
0.03	++	++++	++	++++	+	+++	−	−
0.01	+++	++++	+++	++++	+++	++++	−	−
0.003	+	+++	+	++++	+	++++	−	−
0.001	−	+	−	++	−	+	−	−
0	−	−	−	−	−	−	−	−

For symbols compare Table IV.
α = precipitation after 10 minutes.
β = precipitation after 4 hours.

kept at 37°C. for 2 hours and subsequently for the same period at ice box temperature. The resulting precipitation (α) after 10 minutes and (β) after 4 hours is recorded in Table VII.

The absorbed Type I antiserum still reacted with the acetyl polysaccharide, but not with the de-acetylated product, thus confirming the statements of Avery and Goebel. However, there was no difference between the acetyl polysaccharide treated with effective and with inactivated feces filtrate respectively (nor with the untreated control). The reactivity of all three specimens toward the absorbed Type I antiserum was almost identical, in spite of the fact that the first

specimen had almost completely lost its reactivity against the group specific A-antiserum.

DISCUSSION

In a comparison of the specific carbohydrates of the three main types of pneumococcus, the acetyl polysaccharide of Type I is highest in reactivity towards antisera obtained from rabbits by immunization against human blood corpuscles of group A. The close relationship between this bacterial antigen and the iso-agglutinogen A can be demonstrated by a number of methods. The acetyl substance exerts an inhibitory influence on sheep cell hemolysis by group A-antiserum up to a dilution of about 1:1,000,000 of its 1 per cent solution when especially titrated with optimal dilutions of complement and antiserum. Thus, the bacterial product proves to be almost as active toward the A-antiserum as the carbohydrate of Freudenberg and Eichel which acts in amounts of 1/100 to 1/200γ. Like Freudenberg and Eichel's carbohydrate, isolated from urine of human blood group A subjects, it inhibits the corresponding iso-agglutination. However, in this type of test the necessary amount of the pneumococcus polysaccharide was found to be much higher than in the hemolysis inhibition test, in agreement with observations made by Jorpes on Freudenberg and Eichel's carbohydrate (7).

There is still another method suitable to prove the existence of A-antigen in cells and tissues. Active immunization of rabbits with material containing this antigen, may result in the production of group specific A-antibodies. Only a certain percentage of rabbits, namely the individuals lacking the A-antigen in their organs, are able to produce A-antibodies. In these, one may expect that immunization against Pneumococcus Type I could simultaneously produce antibodies against the A-like antigenicity, observed by us in the genuine carbohydrate of this type. Only small amounts of six individual Pneumococcus Type I antisera (rabbit) were at our disposal, two of which contained group specific A-antibodies. However, since the normal A-antibody in serum of rabbits, lacking A-antigen in their body, would increase also upon any non-specific antigenic irritation, our observations do not permit the assumption that the moderate amounts of group specific A-antibodies present are due to a specific

immunizing effect. Further experiments will be necessary in order to elucidate this point (*cf.* 4).

Of the other type specific carbohydrates, that of Type III was approximately 100 times less effective than the acetyl derivative of Type I in the hemolysis inhibition test, and about 20 times less effective as concerns complement fixation. In the iso-agglutination inhibition test no action by polysaccharide, Type III, could be detected at all. Type II, in turn, was much weaker than Type III in the hemolysis inhibition test. Unless the cross-relationship between the antigen of Pneumococcus Type I and human blood group A is to be considered a fortuitous one, the much lower reactivity of the carbohydrates II and III in our experiments opens the question whether more potent antigens may be derived in the future from these types, in this respect like the acetyl polysaccharide from Type I of Avery and Goebel.

Since the de-acetylated derivative was recognized by Avery and Goebel as an artefact, many earlier contradictory statements regarding its chemical and immunological qualities have found an explanation; nevertheless, the complete absence of reactivity in the present experiments of the de-acetylated product is surprising. The question arose whether the effect of the blood group enzyme of feces filtrates upon the acetyl polysaccharide, Type I, and on the group A substance, which according to Freudenberg and Eichel carries acetyl groups, consists in de-acetylation. In this case, acetyl polysaccharide Type I, treated with feces filtrate, should behave like the de-acetylated product. According to the result of Experiment 7, this is not the case, and hence we have to conclude that the change brought about by this treatment is not identical with or analogous to the de-acetylation of the acetyl polysaccharide.

SUMMARY

1. A relationship between the soluble specific substances of pneumococci and the blood group substance A of man can be demonstrated by the inhibition of sheep cell hemolysis by a group specific A-antiserum. However, there are quantitative differences between the various types.

2. A striking difference exists between the acetyl and the de-acety-

lated polysaccharide of Pneumococcus Type I: The de-acetylated carbohydrate fails to react with the group specific A-antiserum, while the acetyl carbohydrate shows a strong reactivity.

3. The minimum amount of the acetyl polysaccharide, which inhibits sheep cell hemolysis by A-antiserum, is almost as small as that of the group specific carbohydrate isolated by Freudenberg and Eichel from urines of group A.

4. The reactivity of the acetyl polysaccharide can be demonstrated not only by the hemolysis inhibition test, but also by complement fixation and by inhibition of group specific iso-agglutination.

5. Feces filtrates, which possess the ability to destroy the blood group specific substances A and B of man, also affect the acetyl polysaccharide of Pneumococcus Type I. After incubation with an effective feces filtrate, the acetyl polysaccharide almost completely loses its potency toward the group specific A-antiserum and also its ability to inhibit the iso-agglutination of A blood cells.

6. Acetyl polysaccharide of Pneumococcus Type I, having lost its reactivity toward the group-specific A-antiserum after treatment with feces filtrate, still reacts with Type I pneumococcus antiserum which was previously absorbed with de-acetylated polysaccharide, Type I. Thus, the essential effect of the feces filtrate on acetyl polysaccharide, Type I, is not the cleavage of acetyl group, but some other chemical alteration.

Our thanks are due to Dr. M. Heidelberger and to Dr. W. F. Goebel for specimens of some of the polysaccharides, and to Dr. S. D. Beard and Miss F. Clapp of the Lederle Laboratories for pneumococcus antisera.

BIBLIOGRAPHY

1. Schiff, F., and Adelsberger, L., *Z. Immunitätsforsch.*, 1924, **40,** 335. Witebsky, E., *Ergebn. Physiol.*, 1932, **34,** 271.
2. Baily, G. H., and Shorb, M. S., *Am. J. Hyg.*, 1931, **13,** 831.
3. Eisler, M., and Howard, A., *Z. Immunitätsforsch.*, 1932, **76,** 461.
4. Baily, G. H., and Shorb, M. S., *Am. J. Hyg.*, 1933, **17,** 329.
5. Landsteiner, K., *Science*, 1932, **76,** 351. Landsteiner, K., and Levene, P. A., *J. Immunol.*, 1925, **14,** 81.
6. Brahn, B., Schiff, F., and Weinmann, F., *Klin. Woch.*, 1932, **11,** 1592.
7. Freudenberg, K., and Eichel, H., *Ann. Chem.*, Berlin, 1934, **510,** 240.

8. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1933, **58,** 731.
9. Schiff, F., and Akune, M., *Münch. med. Woch.*, 1931, **78,** 657.
10. Schiff, F., and Weiler, G., *Biochem. Z.*, 1931, **235,** 454; **239,** 489.
11. Schiff, F., Über die gruppenspezifischen Substanzen, Jena, Gustav Fischer, 1931.
12. Witebsky, E., and Satoh, T., *Klin. Woch.*, 1933, **12,** 948.
13. Satoh, T., *Klin. Woch.*, 1934, **13,** 798.
14. Sievers, O., *Klin. Woch.*, 1934, **13,** 1640.