THE IMMUNOLOGICAL RELATIONSHIP OF THE CAPSULAR POLYSACCHARIDE OF TYPE XIV PNEUMOCOCCUS TO THE BLOOD GROUP A SPECIFIC SUBSTANCE

By PAUL B. BEESON, M.D., and WALTHER F. GOEBEL, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, June 14, 1939)

Human erythrocytes of all four groups are agglutinated in high titer by Type XIV antipneumococcus horse serum. Finland and Curnen (1), who first noted this action, believe it to be the cause of certain untoward reactions which occur following the therapeutic administration of Type XIV serum. No other type of antipneumococcus horse serum agglutinates erythrocytes in comparable titer; nor does any type of antipneumococcus rabbit serum, even that of Type XIV. In an effort to explain the phenomenon the specific polysaccharide of the Type XIV Pneumococcus has been isolated and its chemical and immunological properties have been investigated (2). There is a striking resemblance between this bacterial carbohydrate and the specific substance of blood group A obtained from commercial peptone. Both substances are nitrogenous polysaccharides constituted from molecules of galactose and acetylglucosamine. The two carbohydrates resemble each other in optical rotation, elementary analysis, and in their precipitation reactions with the salts of heavy metals. The blood group A substance, however, appears to contain nitrogenous constituents which are not present in the pneumococcus carbohydrate.[1] The present communication deals with the immunological properties of these two substances and their relation to the agglutination of human erythrocytes by Type XIV antipneumococcus horse serum.

A detailed comparison of the Type XIV pneumococcus polysaccharide is possible only with the blood group A specific substance, since the chemical nature of the specific substances of the other blood groups has not as yet been sufficiently well characterized. From the information available at present, however, one may assume that the specific substances in the erythrocytes of B and O groups are also carbohydrate in nature (3).

[1] Personal communication from Dr. Karl Landsteiner.

EXPERIMENTAL

Materials

Antisera.—Type XIV antipneumococcus horse serum was obtained through the courtesy of Dr. Augustus Wadsworth and Dr. Harold Lyall of the New York State Department of Health. The corresponding antipneumococcus rabbit serum was produced in these laboratories, using the technique of Goodner, Horsfall, and Dubos (4).

Erythrocytes.—Human erythrocytes of A, B, and O groups were obtained throughout the experimental work from the same three donors. The bloods were preserved in Rous-Turner solution (5 cc. of 5.4 per cent dextrose and 2 cc. of 3.8 per cent sodium citrate for each 3 cc. of whole blood). Erythrocytes were washed three times in normal saline before use.

Specific Substances.—Type XIV pneumococcus specific polysaccharide was isolated from autolysates of whole bacteria grown in meat infusion broth containing peptone and dextrose. The group A specific substance was isolated from commercial peptone. (5). Although probably not identical with the A hapten which occurs in erythrocytes, this substance inhibits the agglutination of human erythrocytes of group A by the serum of group B or O, and also inhibits the lysis of sheep erythrocytes by anti-A rabbit serum. It appears to be identical with the A substance obtained by Landsteiner and Chase (6) from commercial pepsin.

Methods

Absorption of Type XIV Antipneumococcus Serum.—Absorption of the immune serum was accomplished by adding an equal volume of a solution of the Type XIV polysaccharide or group A specific substance to a measured quantity of serum and allowing the mixture to stand overnight at 0°. The optimal concentration of each carbohydrate was predetermined by the method of Sabin (7). The mixture was centrifuged at 0° and the supernatant liquid removed and used for subsequent tests.

Absorption of antipneumococcus serum by erythrocytes required the use of large volumes of washed packed cells. After the cells and serum were mixed and allowed to stand several hours at 0°C., the mixture was centrifuged, and the supernatant fluid removed. This procedure was repeated until no further agglutination of cells by the serum could be detected.

Agglutination of Erythrocytes.—One drop of a 2 per cent suspension of erythrocytes in saline was mixed with one drop of serum dilution and one drop of normal saline in a 7 × 70 mm. agglutination tube. The mixture was allowed to stand for 1 hour at room temperature. The extent of agglutination was estimated by examining a drop of the mixture under low power magnification of the microscope. The dilutions recorded in the protocols refer to the initial dilution of serum used; the final titer was actually about three times that listed. Test for inhibition of agglutination of erythrocytes was performed similarly except that one drop of a saline solution of the inhibiting agent (0.2 per cent A substance; 5 per cent glucosamine or galactose) was substituted for the drop of plain saline.

Inhibition of Lysis of Sheep Erythrocytes by Anti-A Rabbit Serum.—This test was performed according to the technique described by Landsteiner (8). The hemolytic serum was prepared by immunizing rabbits with human erythrocytes of group A. Seven

rabbits were given three courses of four daily intravenous injections, each containing 0.2 cc. of packed erythrocytes. Of the seven rabbits only two developed sheep cell lysins in high titer. The serum used was obtained from these two rabbits.

RESULTS

Inhibition of Lysis of Sheep Erythrocytes.—Although group A substance and Type XIV pneumococcus polysaccharide resemble one another chemically, they differ sharply in their ability to inhibit the lysis of sheep erythrocytes by anti-A rabbit serum in the presence of complement. Group A substance is active when present in a concentration of 1:8,000,000 whereas the bacterial polysaccharide has no inhibiting action even in concentrations up to the limit of its solubility, which is about 1 per cent.

Precipitation of Type XIV Pneumococcal Polysaccharide and Group A Substance in Type XIV Antipneumococcus Sera.—Type XIV pneumococcal polysaccharide precipitates in homologous antipneumococcus serum, either horse or rabbit, in dilutions as great as one part in four million. The reaction is essentially independent of temperature variation between 0° and 37°C. The group A substance also precipitates in Type XIV horse serum, in dilutions as high as one part in a million. The latter reaction occurs only at or near 0°C., however; the precipitate thus formed redissolves if the temperature is raised to 20°C. or higher. No precipitation of the A substance occurs in Type XIV rabbit serum, irrespective of the dilution or the temperature at which the reactions are carried out as can be seen from Table I. The difference in the precipitation reactions of Type XIV antipneumococcus horse and rabbit sera parallels the difference in their action on human erythrocytes, which are agglutinated by Type XIV horse serum, but not by Type XIV rabbit serum (1).

Absorption of Type XIV Antipneumococcus Horse Serum by Polysaccharides.—After absorption with the homologous bacterial polysaccharide Type XIV horse serum fails to precipitate with the group A substance as seen from the results presented in Table II. On the other hand, complete absorption of the same serum with A substance seems to cause no gross qualitative change in the precipitation with the Type XIV specific polysaccharide. By quantitative methods, however, it has been possible to demonstrate that absorption with A substance removes approximately 50 per cent of the antibody nitrogen specifically precipitable with the Type XIV carbohydrate (2).

The lack of complete reciprocal absorption of precipitin is in accordance with the observations of Finland and Curnen, who found that absorption of Type XIV antiserum with homologous bacterial cells removed all hemag-

glutinins while absorption with human erythrocytes did not appreciably diminish the titer of specific agglutinins for the homologous type specific organisms.

TABLE I

Precipitation of Group A Substance in Type XIV Antipneumococcus Horse and Rabbit Sera

Type XIV antipneumococcus serum	Temperature	Final dilution of A substance			
		1-10,000	1-50,000	1-250,000	1-1,000,000
	°C.				
Horse	0	+++	++	+	±
	37	0	0	0	0
Rabbit	0	0	0	0	0
	37	0	0	0	0

++++ = marked precipitation.
+++ = turbidity and granular precipitation.
++ = turbidity and fine precipitation.
+ = turbidity.
± = faint cloud.
0 = no precipitation.

TABLE II

Precipitation Reactions of Type XIV Antipneumococcus Horse Serum before and after Absorption with Specific Substances

Type XIV antipneumococcus horse serum	Precipitinogen	Final dilution of precipitinogen				
		1-10,000	1-50,000	1-250,000	1-1,000,000	1-4,000,000
Unabsorbed	XIV polysaccharide	++++	++++	+++	+±	±
	A substance	+++	++	+	±	0
Absorbed with A substance	XIV polysaccharide	++++	+++±	++±	+	±
	A substance	0	0	0	0	0
Absorbed with XIV polysaccharide	XIV polysaccharide	0	0	0	0	0
	A substance	0	0	0	0	0

Agglutination of Erythrocytes in Type XIV Antipneumococcus Serum.—Table III illustrates the agglutinating action of Type XIV horse serum on human erythrocytes of A, B, and O groups. A noteworthy point is the fact that there is no significant difference in the titers of agglutinins for the cells of different blood groups. In addition it can be seen that when Type XIV horse serum is absorbed with the homologous bacterial polysaccharide or with the group A substance there is a marked change in its

capacity to agglutinate human erythrocytes. Absorption with the former completely removes the agglutinins for erythrocytes. Absorption with A substance, although not removing the agglutinins completely, diminishes the action of the serum markedly. It should be noted, furthermore, that absorption with A substance reduces the agglutinins not only for erythrocytes of the same group but also for those of B and O groups as well. The reduction is evident not only in the final titer, but also in the strength of

TABLE III

Agglutination of Human Erythrocytes by Type XIV Antipneumococcus Horse Serum before and after Absorption with Specific Substances

Type XIV antipneumococcus horse serum	Erythrocytes of group	Serum dilution used				
		1-10	1-20	1-40	1-80	1-160
Unabsorbed	A	++++	+++±	+++	++	+
	B	++++	+++±	++±	++	+
	O	++++	+++±	+++	+±	+
Absorbed with XIV polysaccharide	A	0	0	0	0	0
	B	0	0	0	0	0
	O	0	0	0	0	0
Absorbed with A substance	A	++	+±	+	+	0
	B	++±	++	+	±	0
	O	++	+±	±	±	0

++++ = complete agglutination.
+++ = strong agglutination.
++ = partial agglutination.
+ = detectable agglutination.
± = questionable agglutination.
0 = no agglutination.

agglutination at lower dilutions of serum. An interesting variation of the above experiment is one in which A substance (0.2 per cent in saline) is added to the agglutinating system at room temperature as seen in Table IV. At this temperature there is no visible precipitation between A substance and Type XIV horse serum. Nevertheless, there seems to be combination with at least a portion of the antibody, for the agglutination titer is diminished to a degree comparable with that found in the A-absorbed serum. Neither glucosamine nor galactose, the hexoses from which the bacterial and blood group polysaccharides are constituted, have an inhibiting action on the agglutination of the erythrocytes of Type XIV horse serum.

Absorption of Type XIV Antipneumococcus Horse Serum with Erythrocytes.—Absorption of the hemagglutinins in Type XIV horse serum with erythrocytes of each of the three groups requires relatively large quantities of cells. As was observed by Finland and Curnen, absorption with the erythrocytes of one group removes the agglutinins not only for that group,

TABLE IV

Inhibition of Agglutination of Human Erythrocytes in Type XIV Antipneumococcus Horse Serum by A Substance

Inhibiting agent	Erythrocytes of group	Serum dilution used				
		1-10	1-20	1-40	1-80	1-160
None	A	++++	+++±	+++	++	+
	B	++++	++++	++±	++	+
	O	++++	+++±	+++	+±	+
A substance	A	++±	+±	+	+	0
	B	++±	+±	+	+	0
	O	+++	++	+	±	0

TABLE V

Precipitin Reactions of Type XIV Antipneumococcus Horse Serum before and after Absorption with Human Erythrocytes

Type XIV antipneumococcus rabbit serum	Precipitinogen	Final dilution of precipitinogen				
		1-10,000	1-50,000	1-250,000	1-1,000,000	1-4,000,000
Unabsorbed	Type XIV polysaccharide	++++	++++	+++	+±	±
	A substance	+++	++	+	±	0
Absorbed with erythrocytes of group A, B, or O	Type XIV polysaccharide	++++	+++±	++±	+	±
	A substance	+±	±	0	0	0

but also for the other two groups. This fact seems to point to the presence of similarly reacting substances in the erythrocytes of the different groups.

The precipitation reactions of Type XIV polysaccharide and A substance in the Type XIV horse serum absorbed with erythrocytes of the various blood groups are shown in Table V. It will be observed that there is no appreciable change in the precipitin reaction with Type XIV polysaccharide, whereas precipitation with A substance is markedly reduced. Furthermore, the diminution of precipitins for A substance is the same regardless of the group of erythrocytes used in the absorption.

DISCUSSION

The development of lysins or agglutinins for the erythrocytes of different animal species following immunization with bacterial cells has been noted on a number of occasions in the past. For example, immunization of rabbits with bacteria containing Forssman antigen causes the development of lysins and agglutinins for the erythrocytes of sheep. Buchbinder (9) lists more than twenty bacterial species which are known to contain Forssman antigen, the Pneumococcus being one of these. Eisler (10) observed that immunization of rabbits with paratyphoid B organisms resulted in the formation of agglutinins for human erythrocytes of group A. He also reported that immunization of goats with Shiga bacilli caused the production of agglutinins for human erythrocytes of all four groups; although this phenomenon did not occur when rabbits or horses were so immunized. Buchbinder (11) found that immunization of the rabbit, guinea pig, rat, or monkey with bacteria belonging to the hemorrhagic septicemia group evoked the production of lysins and agglutinins for the erythrocytes of many varieties of birds.

It would seem that the special case with which this communication is concerned, that is, the agglutination of human erythrocytes by the sera of horses immunized with Type XIV pneumococci, may be regarded as another example of a heterogenetic relationship. The fact that this relationship is between horse serum, the serum of an animal which contains the Forssman antigen, and erythrocytes of all four human blood groups would make it improbable that it belongs to the Forssman classification. It is well known that human erythrocytes of groups B and O do not contain Forssman antigen.

The presence in Type XIV antipneumococcus horse serum of antibodies reactive with human erythrocytes in contrast to their absence in the corresponding antiserum of the rabbit, is evidence of differences in the immune response of different animal species. Weil and Sherman (12) call attention to the fact that rabbit erythrocytes are also agglutinated by Type XIV antipneumococcus horse serum. They infer from this that in the erythrocytes of the rabbit there is present an antigen which resembles that in human erythrocytes and this species is therefore incapable of evoking agglutinins for human cells. There can be little doubt that the erythrocyte-agglutinating antibodies in Type XIV horse serum are the specific anti-carbohydrate immune bodies evoked by the encapsulated Type XIV pneumococcus for they can be removed entirely by absorption with the Type XIV specific polysaccharide. It appears therefore that the phenom-

enon of erythrocyte agglutination is a cross-reaction between the type specific anticarbohydrate immune bodies of the horse serum and the human blood cells. Cross-reactions between pneumococcal types are notably more common with horse antisera than with rabbit antisera. White (13) states that at the Massachusetts Antitoxin and Vaccine Laboratory it was observed that even when animals were immunized with the same vaccines horse antisera often gave cross-reactions, while rabbit antisera tended to be type specific. In this laboratory we have shown that certain artificial antigens containing hexuronic acids and even benzene sulfonic and carboxylic acids precipitate with antipneumococcal horse sera of various types (14). Such antigens, however, show no precipitation reaction in the corresponding immune rabbit sera. Heidelberger and Kendall (15) found that Type III antipneumococcus horse sera precipitated certain of the hydrolytic products of the Type III pneumococcus polysaccharide, whereas Type III antipneumococcus rabbit serum did not. These investigators (16) also showed by quantitative methods that antipneumococcal horse sera Types III and VIII gave extensive cross-precipitation reactions with the heterologous bacterial carbohydrates, while rabbit antisera of these same types reacted almost exclusively with the homologous polysaccharides. In the present instance it is shown that the A substance precipitates with the anticarbohydrate immune bodies of Type XIV horse serum, but not at all with those of the corresponding rabbit antiserum.

The serological relationship between the Type XIV and group A specific substances appears to be correlated with similarities in the chemical constitution of these specific polysaccharides. Differences in their serological behavior as demonstrated by the lack of reciprocal absorption, strongly support the chemical evidence of similarity rather than identity of the specific substances. It should be emphasized here that the "group A substance" used in these experiments is probably not identical with the blood group A hapten as it occurs naturally in the intact human erythrocyte. The structure of the specific substances of the other blood groups is as yet insufficiently established to permit comparison with the group A and the Type XIV pneumococcal polysaccharides. It may be that some common grouping in the blood group substances and the Type XIV polysaccharide is responsible for the interaction of Type XIV antipneumococcus horse serum and human erythrocytes of all groups.

SUMMARY

1. The agglutination of human erythrocytes and the precipitation of the blood group A substance by Type XIV antipneumococcus horse serum

are properties of the specific anticarbohydrate immune bodies in the serum.

2. Absorption of Type XIV antipneumococcus horse serum with the homologous bacterial polysaccharide removes the agglutinins for human erythrocytes as well as the precipitins for the group A substance.

3. Absorption of Type XIV antipneumococcus horse serum with the group A substance markedly diminishes the ability of the serum to agglutinate erythrocytes of all groups.

4. Absorption of Type XIV antipneumococcus horse serum with human erythrocytes causes a marked diminution in the precipitation with group A substance.

5. The chemical and immunological relationship between the specific substances of blood group A and the Type XIV Pneumococcus is discussed.

BIBLIOGRAPHY

1. Finland, M., and Curnen, E. C., *Science*, 1938, **87**, 417.
2. Goebel, W. F., Beeson, P. B., and Hoagland, C. L., *J. Biol. Chem.*, 1939, **129**, 455.
3. Hallauer, C., *Z. Immunitätsforsch.*, 1934, **83**, 114.
4. Goodner, K., Horsfall, F. L., Jr., and Dubos, R. J., *J. Immunol.*, 1937, **33**, 279.
5. Goebel, W. F., *J. Exp. Med.*, 1938, **68**, 221.
6. Landsteiner, K., and Chase, M. W., *J. Exp. Med.*, 1936, **63**, 813.
7. Sabin, A. B., *J. Exp. Med.*, 1931, **53**, 93.
8. Landsteiner, K., *J. Exp. Med.*, 1936, **63**, 185.
9. Buchbinder, L., *Arch. Path.*, 1935, **19**, 841.
10. Eisler, M., *Z. Immunitätsforsch.*, 1931, **73**, 37.
11. Buchbinder, L., *J. Immunol.*, 1934, **26**, 215.
12. Weil, A. J., and Sherman, E., *J. Immunol.*, 1939, **36**, 139.
13. White, B., Biology of pneumococcus, New York, Commonwealth Fund, 1938, 594.
14. Goebel, W. F., and Hotchkiss, R. D., *J. Exp. Med.*, 1937, **66**, 191.
15. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1933, **57**, 373.
16. Heidelberger, M., Kabat, E. A., and Shrivastava, D. L., *J. Exp. Med.*, 1937, **65**, 487.