A NOTE ON THE SPECIFIC AGGLUTINATION OF PNEUMOCOCCUS TYPES I, II AND III

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(Received for publication, November 13, 1931)

In a preceding paper we presented evidence for the existence of a type-specific substance (*i.e.* the so called A substance) in Pneumococcus Type I distinct from the specific carbohydrate of Avery and Heidelberger. During the course of the experiments described in that communication it was noted that untreated Antipneumococcus Type I rabbit sera which contained no precipitin against the purified carbohydrate, nevertheless exhibited agglutinin titre against the homologous organism at least as high as 1/240. This observation suggested the possibility that the S organism might contain an agglutinogen unrelated to the specific carbohydrate. Moreover, since the same antisera were found to contain precipitin reacting with the type-specific A substance, the hypothesis that the observed agglutination might also be caused by the A antibody seemed not unreasonable. The experiments to be described not only lend further support to this hypothesis, but confirm the original observation concerning the agglutination of Pneumococcus Type I by antisera which fail to react by visible precipitation with the specific carbohydrate as well as extend these findings to the smooth organisms of Types II and III.

Technique

Preparation of the Antigens.—Cultures of pneumococci Types I, II and III which had been recently passed through mice were grown in phosphate buffer hormone broth. The growth after incubation at 37°C. for 18 hours was removed by centrifuging and resuspended in a volume of physiological salt solution equivalent to that of the decanted broth. The saline suspension was then divided into three portions. To the first, sufficient formalin was added to give a concentration of 0.2 per cent. The formalinized suspension was then maintained at 37°C. for 14 to 16 hours.

The reaction of the second portion of the saline suspension was adjusted to about pH 5 by means of acetic acid and subsequently maintained at 100°C. in the Arnold for 40 minutes. The acidified suspension was allowed to stand at 37°C. for 14 to 16 hours. The pH was then brought to 7 with NaOH.

The third portion was treated with NaOH until a pH of about 8.8 was obtained. It was thereupon subjected to the same physical conditions as the acidified suspension, with final adjustment of the pH to 7.

In most of the experiments these suspensions were employed within a day or two of preparation. In a few cases older antigen solutions were used which had been kept in the ice box for from 1 to 2 weeks.

Examination of smears of the heated suspensions stained by Gram's method revealed no gross changes in the typical morphology of the organism, nor was its ability to retain the gentian violet impaired.

Preparation of Antisera.—The antipneumococcus horse sera were typing sera secured in the cases of Type I and Type II from the Massachusetts State Antitoxin Laboratory and in the case of Type III from the New York State Laboratory at Albany.

The type-specific antipneumococcus rabbit sera were produced by injection of rabbits with formalinized suspensions of pneumococci. These untreated antisera will hereafter be referred to as "unabsorbed."

The antisera which in this paper are designated as "absorbed" were obtained by repeated precipitation of the type-specific unabsorbed antisera described above with the homologous specific carbohydrate purified according to the method of Heidelberger and his associates. The details of the technique employed in the removal of the S antibody are described in a previous communication (1).

A comparison of the precipitating power of the horse antisera used in the following experiments before and after absorption with the carbohydrate is shown in Table I. The absorbed rabbit sera used in the experiments with Pneumococcus Type I and Type II exhibited the same inability to precipitate in the presence of the specific carbohydrate.

EXPERIMENTAL

The experiments which indicated the presence of a type-specific agglutinogen in Pneumococcus Types I, II and III distinct from the specific carbohydrate consisted simply in testing the three antigens prepared in the manner already described against the homologous unabsorbed or untreated antiserum and the same antiserum from which the antibody reacting with the specific carbohydrate had been largely removed by repeated precipitation at the optimum point of flocculation with the

TABLE I

Effect of Absorbing Pneumococcus Antisera with the Specific Carbohydrate

Dilution of the specific carbohydrate		1/100	1/1,000	1/10,000	1/100,000	1/1,000,000	1/2,000,000	1/4,000,000	1/8,000,000
Antiserum	Antigen								
Antipneumococcus I horse serum unabsorbed	Specific carbohydrate Pneumococcus I	++++	++++	++++	++++	++	+	+	
Antipneumococcus I horse serum absorbed	Specific carbohydrate Pneumococcus I	±?	−	−	−	Not done	Not done	Not done	
Antipneumococcus II horse serum unabsorbed	Specific carbohydrate Pneumococcus II	++++	++++	++++	++++	+	+	.−	
Antipneumococcus II horse serum absorbed	Specific carbohydrate Pneumococcus II	±?	−	−	−	−	−	−	
Antipneumococcus III horse serum unabsorbed	Specific carbohydrate Pneumococcus III	++++	++++	++++	++++	++	+±	+	
Antipneumococcus III horse serum absorbed	Specific carbohydrate Pneumococcus III	±	−	−	−	−	−	−	

The results recorded above were those obtained from ring tests read after standing 2 hours at room temperature.

TABLE II

Agglutination of Pneumococcus I in Unabsorbed and Absorbed Antisera

Dilution of the serum		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/312	1/1,024
Antiserum	Antigen										
Antipneumococcus I horse serum unabsorbed	Formalinized Pneumococcus I	++++	++++	++++	++++	++++	++++	+++	+±	−	−
Antipneumococcus I horse serum absorbed	Formalinized Pneumococcus I	++++	++++	++++	++++	++++	+++±	++	+	−	−
Antipneumococcus I horse serum unabsorbed	Pneumococcus I boiled pH 5	++++	++++	++++	++++	++++	+++±	++	+	−	−
Antipneumococcus I horse serum absorbed	Pneumococcus I boiled pH 5	++++	++++	++++	++++	++++	++±	+	±	−	−
Antipneumococcus I horse serum unabsorbed	Pneumococcus I boiled pH 8.8	++++	++++	++++	++++	++++	+++	+±	+	±	−
Antipneumococcus I horse serum absorbed	Pneumococcus I boiled pH 8.8	+++±	+	−	−	−	−	−	−	−	−
Normal horse serum	Formalinized Pneumococcus I	±	±	+	±?	−	−				
Normal horse serum	Pneumococcus I boiled pH 5	±	−	−	−	−	−				
Normal horse serum	Pneumococcus I boiled pH 8.8	±	−	−	−	−	−				

Tubes in agglutination tests contained equal parts of diluted antiserum and antigen suspension. Readings after 16 hours in water bath at 53°C.

purified polysaccharide. To show that the agglutination obtained with the absorbed antisera was type-specific, cross-agglutination tests with the heterologous absorbed antisera were performed. No agglutination of the heterologous organism was observed in the presence of these sera.

In Table II are recorded the results of an experiment in which the agglutinin titre of Type I pneumococcus unabsorbed and absorbed antisera were determined in the presence of suspensions of Pneumo-

TABLE III

Effect of Heating the Specific Carbohydrates of Pneumococcus I, II and III at pH 8.8

Dilution of type-specific antiserum	1/10,000	1/100,000	1/1,000,000	1/2,000,000	1/4,000,000	1/8,000,000
Antigen						
Pneumococcus I specific carbohydrate unboiled	++++	++++	++	+	±	±?
Pneumococcus I specific carbohydrate boiled at pH 8.8	++++	++++	+±	+	±	−
Pneumococcus II specific carbohydrate unboiled	++++	+++±	+	+	−	−
Pneumococcus II specific carbohydrate boiled at pH 8.8	++++	+++±	+±	±	−	−
Pneumococcus III specific carbohydrate unboiled	++++	++++	++	+±	+	±
Pneumococcus III specific carbohydrate boiled at pH 8.8	++++	++++	++±	++	+	±

Ring test readings after 2 hours at room temperature.

coccus Type I after formalinization and after boiling at acid and alkaline reactions. The experiment was repeated using an Anti-pneumococcus I rabbit serum. Results of identical character were obtained.

A comparison of the titres of the unabsorbed and absorbed sera show that (1) the removal of a large proportion of the specific carbohydrate-precipitating antibody does not reduce the agglutinating titre of the serum for Pneumococcus Type I when treated with formalin or boiled at pH 5, and (2) that when this organism is boiled at pH

TABLE IV

Agglutination of Pneumococcus II in Unabsorbed and Absorbed Antisera

Dilution of the serum		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560
Antisecum	Antigen									
Antipneumococcus II horse serum unabsorbed	Formalinized Pneumococcus II	++++	++++	++++	++++	+++±	+	−	−	−
Antipneumococcus II horse serum absorbed	Formalinized Pneumococcus II	++	++±	+±	+	+	±	−	−	−
Antipneumococcus II horse serum unabsorbed	Pneumococcus II boiled pH 5	++++	++++	++++	++++	+++±	++	±	−	−
Antipneumococcus II horse serum absorbed	Pneumococcus II boiled pH 5	+++	++++	+++	+±	+	±	−	−	−
Antipneumococcus II horse serum unabsorbed	Pneumococcus II boiled pH 8.8	++++	++++	++++	++++	+++	±?			
Antipneumococcus II horse serum absorbed	Pneumococcus II boiled pH 8.8	++++	+++	+	±?					
Antipneumococcus II rabbit serum unabsorbed	Formalinized Pneumococcus II	++++	++++	+++	++	+±	+	+	±	−
Antipneumococcus II rabbit serum absorbed	Formalinized Pneumococcus II	+++	+++	++±	++	++	+±	+	±	−

Antipneumococcus II rabbit serum unabsorbed	Pneumococcus II boiled pH 5	++++	++++	+++	++	+±	+	±	−	−
Antipneumococcus II rabbit serum absorbed	Pneumococcus II boiled pH 5	++±	++	+++	++	++	+	±	−	−
Antipneumococcus II rabbit serum unabsorbed	Pneumococcus II boiled pH 8.8	++++	++++	++±	++	−	−	−	−	−
Antipneumococcus II rabbit serum absorbed	Pneumococcus II boiled pH 8.8	±	−	−	−	−	−	−	−	−
Antipneumococcus II rabbit serum unabsorbed	Pneumococcus II specific carbohydrate	+++	++	+	±					

Tubes in agglutination tests contained equal parts of diluted antiserum and antigen suspension. Readings after 16 hours in water bath at 50°C.

Controls consisting of dilutions of normal horse and rabbit sera were negative.

8.8, it fails to be agglutinated by the absorbed serum. That boiling at this reaction does not entirely destroy its capacity to act as an agglutinable particle in the presence of antiserum containing the S-precipitating antibody is also made clear.

Since, as is shown in Table III, the purified specific carbohydrates of the three pneumococcus types after boiling for 40 minutes at a reaction of pH 8.8 show no definite reduction of their function as precipitating agents, it is reasonable to infer that the constituent in Pneumococcus Type I responsible for the agglutination of the formalinized or acid-boiled organism in the absorbed antiserum and which is rendered inactive by boiling at pH 8.8 is a type-specific agglutinogen differing essentially from the specific carbohydrate.

Employing the same technique, the experiment was again performed using as antigens suspensions of Pneumococcus Type II. The results given in Table IV parallel those obtained for Pneumococcus Type I. Pneumococcus Type II also apparently contains an agglutinogen which is heat-labile at an alkaline reaction.

It will be noted that in the case of Pneumoccoccus Type I the titre of the unabsorbed antiserum was the same for all three suspensions. In the Type II unabsorbed rabbit antiserum the end-point of the titration in which the alkaline-boiled suspension is used falls short of that recorded for the formalinized and acid-boiled antigen. This fact affords further evidence of the presence of an agglutinogen distinct from the specific carbohydrate, since it is probable that the end-point of the alkaline-boiled suspension represents the final effect of the antibody reacting with the specific carbohydrate; while the end-points of the formalized and acid-boiled suspension indicate the quantity at which a second type-specific agglutinin ceases to react. A certain amount of experimental support is given to this hypothesis by the precipitating titre obtained by diluting the unabsorbed anti-Type II pneumococcus rabbit serum and using a constant quantity of the purified specific carbohydrate as antigen. The end-point of such a titration, to be found in Table IV, closely approximates that attained when the alkaline-boiled suspension is added to the same dilutions of the antiserum.

In parallel experiments with Pneumococcus Type III the outcome was somewhat different. The data assembled in Table V show that

TABLE V

Agglutination of Pneumococcus III in Unabsorbed and Absorbed Sera

Dilution of serum		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	1/5,120
Antiserum	Antigen										
Antipneumococcus III horse serum unabsorbed	Pneumococcus III formalinized	++++	++++	++++	++++	++++	++++	+++	++	±	−
Antipneumococcus III horse serum absorbed	Pneumococcus III formalinized	++++	++++	++++	++++	++++	++	+	−	−	−
Antipneumococcus III horse serum unabsorbed	Pneumococcus III boiled pH 5	++++	++++	++++	++++	++++	++++	+++	++	±	−
Antipneumococcus III horse serum absorbed	Pneumococcus III boiled pH 5	++++	++++	++++	++++	++++	++++	+++	+	±	−
Antipneumococcus III horse serum unabsorbed	Pneumococcus III boiled pH 8.8	++++	++++	++++	++++	++++	++++	+++	++	±	−
Antipneumococcus III horse serum absorbed	Pneumococcus III boiled pH 8.8	++++	++++	++++	++++	++++	++++	++	+	±	−
Normal horse serum	Pneumococcus III formalinized	+++	++	+±	+	±					
Normal horse serum	Pneumococcus III boiled pH 5	++±	++	+±	+	±					
Normal horse serum	Pneumococcus III boiled pH 8.8	+++	++±	+±	+±	+					

Tubes in agglutination tests contained equal parts of diluted antiserum and antigen suspension. Readings after 16 hours in water bath at 50°C.

whereas this organism behaves like Pneumococcus Types I and II in being agglutinated to the same degree by unabsorbed and absorbed antisera, its susceptibility to agglutination in the absorbed antiserum is not removed by boiling at pH 8.8. Accordingly, it is impossible to assert with the same assurance that the agglutination of this organism in the absorbed antiserum is dependent upon an agglutinogen distinct from the specific carbohydrate. Nevertheless, it appears unlikely that the end-point of agglutination would remain practically unchanged after the removal from the antiserum of a very large proportion of the antibody against the specific carbohydrate if agglutination were dependent exclusively upon the interaction of those entities.

DISCUSSION

The experiments recorded above have shown that type-specific agglutination of Pneumococcus Types I, II and III occurs in homologous antiserum to approximately the same titre after the antibody reacting with the purified specific carbohydrate has been largely removed. This fact strongly suggests that in these organisms there exists a type-specific agglutinogen which is to be distinguished from the specific carbohydrate. The facts that boiling at pH 8.8 greatly reduces the agglutinability of Pneumococcus Type I and Type II in the presence of such absorbed sera, whereas boiling at pH 5 or formalinization leaves these organisms still susceptible to the agglutinating action of absorbed antisera reinforce the evidence for the existence of a type-specific agglutinogen other than the specific carbohydrate.

It has recently been shown (1) that the autolysate of Pneumococcus Type I contains a type-specific precipitating substance which is distinguished most markedly from the specific carbohydrate by its inability to resist heating in alkaline solution. Although the evidence is by no means sufficient to identify this heat-labile precipitating body or A substance with the type-specific agglutinogen described in this paper, the facts that they are both heat-labile in alkaline but not in acid solution and that both occur in antisera largely deprived of the antibody suggest that they may be the same antigenic substance.

Experimental support for a similar type-specific antigen in Pneumococcus Type III is not as adequate as in the cases of Pneumococcus I

and II. The fact, however, that antisera absorbed with the purified carbohydrate agglutinate the organism to the same titre as untreated sera points to the presence of an antigen of this nature.

Two points are perhaps worthy of comment in the light of the possibility of the existence of a type-specific agglutinogen in the pneumococcus unrelated to the specific carbohydrate. The first deals with the standardization of antisera for therapeutic use, such as antimeningococcus sera. At present the sole criterion for the therapeutic efficiency of antimeningococcus serum is a high agglutinin titre. Since it has been shown (2, 3) that at least as far as conferring protection goes in mice against the pneumococcus it is the specific carbohydrate antibody which is by far the most important if not the essential factor, and since these experiments make it clear that antisera may occur after immunization which contain no demonstrable precipitating antibody against that substance, but do contain agglutinins, it becomes evident that if standardization is based only on agglutinin titre, inadequate therapeusis may occasionally result.

The second application of the results of these experiments is to the possible explanation of the facts noted by Avery and his associates (4) that *B. friedlaenderi* Type B failed to absorb the agglutinins from Pneumococcus Type II antiserum, and that Pneumococcus Type II did not remove the agglutinins from *B. friedlaenderi* Type B antiserum, although these authors have shown that the specific carbohydrates derived from these organisms are probably identical.

The presence of a specific agglutinogen in Pneumococcus Type II unrelated to the specific carbohydrate would account for this failure to establish the complete serological identity of the two organisms.

CONCLUSIONS

1. Type-specific agglutination of Pneumococcus Types I, II and III has been demonstrated in antisera largely deprived of the carbohydrate antibody.

2. The type-specific agglutinogens in Pneumococcus I and II responsible for agglutination in such antisera are inactivated by heating in alkaline solution at 100°C. The specific carbohydrate remains unaltered under these conditions.

3. The relationship of the type-specific agglutinogen in Pneumococcus I to the type-specific A substance has been discussed.

4. The possible application of these results to the standardization of therapeutic antisera by agglutinin titre has been discussed.

5. On the basis of the experiments recorded a tentative explanation is offered for the failure of Pneumococcus Type II to absorb the agglutinins from anti-*B. friedlaenderi* Type B serum.

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