THE IDENTITY OF THE MECHANISMS OF TYPE-SPECIFIC AGGLUTININ AND PRECIPITIN REACTIONS WITH PNEUMOCOCCUS

By THOMAS FRANCIS, Jr., M.D.

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

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In the course of some experiments, the serum obtained from a rabbit actively immunized against Type II Pneumococcus was injected intravenously into a normal rabbit. Before injection the serum had been shown to contain type-specific agglutinins for the homologous organism and precipitins for the type-specific polysaccharide derived from organisms of the same type. When the serum of the passively immunized animal was tested 24 hours later, type-specific agglutinins were present but no visible precipitin reaction with the polysaccharide could be demonstrated. Since the haptens are known to produce inhibition phenomena in serological reactions (the type-specific polysaccharides react serologically as haptens), it was conceivable that in the precipitin tests soluble specific substance (S-free) and antibody had combined without producing precipitation. To test this hypothesis, equal amounts of a suspension of homologous heat-killed pneumococci were added to each of a series of tubes which contained equal amounts of immune serum and varying dilutions of the type-specific polysaccharide. In the tubes containing the highest concentration of type-specific polysaccharide little or no agglutination occurred, whereas in the presence of weak concentrations of soluble specific substance the degree of agglutination approached that present in the tubes containing only immune serum.

The specific capsular polysaccharides are known to be the components of the respective types of Pneumococcus which determine their type specificity. These carbohydrates have been obtained by chemical methods in a purified state, completely separated from the bacterial cell. In this state they are readily soluble and react sero-

logically in the precipitin reaction with homologous immune sera. The observations just described indicated that soluble specific substance may exert a definite influence upon the type-specific agglutination reaction. The subsequent work was designed, therefore, to ascertain what this influence might be and, if possible, to correlate the mechanisms of the precipitin and agglutinin reactions.

Materials

1. Type-Specific Polysaccharides.—These were obtained from pneumococci by the method of Heidelberger and Avery (1). The dilutions were made in physiological salt solution.

2. Immune Sera.—Immune rabbit serum was obtained from rabbits immunized by the method of Cole and Moore (2). Immune horse serum was obtained from the New York State Board of Health Laboratories through the courtesy of Dr. A. B. Wadsworth. In general, rabbit serum was found more satisfactory than horse serum in the type of experiment here described, since the titer of antibodies in the former is generally not so high and sharper end-points may be obtained.

EXPERIMENTAL

Experiment 1.—To 5 cc. of antipneumococcus Type I horse serum were added the bacteria recovered by centrifugation from 50 cc. of a Type I pneumococcus culture suspended in 5 cc. of physiological saline. The mixture was placed in a water bath at 37°C. for 2 hours—then in the ice box overnight. The next day the tube was centrifugated and the agglutinated material removed. This mass was mixed with 9.5 cc. of Type I S so as to produce a final concentration of 1:200,000 of the latter. The tube was shaken at 15 minute intervals for 2 hours and then placed in the ice box. After 24 hours the tube was centrifugated and the supernatant fluid removed by pipette. The amount of unbound S in the supernatant fluid was determined. This was done by titration with immune serum, comparing the results with those obtained with known amounts of S. If no change in the concentration of S in the fluid had been produced by exposing it to the agglutinated material the end-point should be the same as that of the control. In fact, however, the control material reacted in a dilution of 1:1,600,000 while the S in the supernatant reacted in a dilution equivalent to only 1:400,000, a decrease to one-quarter the amount originally present.

Let us assume, as suggested by Avery and Heidelberger (3), that type-specific agglutination is produced by the union of antibody with the capsular carbohydrate at the surface of the cell (S-cellular). On this basis the probable explanation of Experiment 1 is that when pneumococci, in numbers insufficient to absorb the serum completely,

are specifically agglutinated, an excess of antibody is present in the combination. This excess can then unite with additional free S forming a complex of antibody with free and cellular S. Since the amount of antibody is constant, the ratio of antibody to S is smaller in the second phase than in the primary agglutination.

In the precipitin reaction an excess of precipitinogen added to the precipitate causes the precipitate to dissolve. To clarify the relation of the type-specific agglutinin and precipitin reactions, therefore, it is necessary to determine the effect of an excess of free S upon the combination of antibody and cellular S in the agglutination reaction.

Experiment 2.—To 0.5 cc. of antipneumococcus Type II rabbit serum was added 0.5 cc. of a concentrated suspension of Type II Pneumococcus. Prompt agglutination occurred and a firm disc formed. Following incubation for 2 hours at 37°C., 0.5 cc. of a 1:200 dilution of Type II soluble specific substance was added. The mixture was agitated and placed in the water bath. After 45 minutes agglutination was no longer detectable but the organisms had become redispersed into their original, suspended state.

The experiment was repeated in a modified manner.

After agglutination had occurred, as above, the disc was removed, washed gently with physiological saline once, and mixed with 0.5 cc. of 1:200 Type II soluble specific substance. The agglutinated disc became redispersed. The organisms were then centrifuged from their state of suspension and resuspended in physiological saline, Type I serum, and Type II serum, respectively. That the organisms were not rendered spontaneously agglutinable by the original agglutination was shown by the fact that they did not agglutinate in the saline or Type I serum. Nor had they lost their agglutinability since they were promptly agglutinated by the Type II serum. The supernatant fluid after centrifugation of the organisms gave a well marked reaction with Type II serum showing that an excess of free S was present.

By the above procedures it was clearly demonstrated that an excess of free S produces a prompt and complete reversal of the agglutinin reaction. With the return of the agglutinated bacteria to a state of suspension they are again specifically agglutinable. Two interpretations of the mechanism of the experimental results suggest themselves. The first is that the antibody originally combined with the cellular S is released from that combination and bound in turn by the free S. The other and more consistent explanation is that the combination of

antibody with cellular S at the surface of the cell forms a soluble product in the presence of the excess free S. In the latter instance, however, only a small amount of the capsular polysaccharide is removed from the cell since it regains a normal agglutinability.

In the preliminary experiments it was noted that an excess of free S prevented agglutination and presumably combined with the entire amount of antibody. The following experiment was designed to show that no detectable antibody is bound by cellular S under these conditions, but rather that it is in firm combination with the free soluble specific substance.

Experiment 3.—Pooled antipneumococcus Type II rabbit serum was used. The agglutinin titer was positive in 1:128 dilution; the precipitin titer was + with 1:800,000 dilution of Type II soluble specific substance; and the serum when diluted above 1:20 gave no precipitin reaction with an optimal concentration of soluble specific substance. To 0.2 cc. of serum was added 0.3 cc. of physiological saline and 0.5 cc. of a 1:200 dilution of Type II soluble specific substance. No precipitation occurred after 2 hours incubation and overnight storage in the ice box. To the tube was added 1 cc. of a concentrated heat-killed suspension of Type II pneumococci. The organisms were not agglutinated. In the control tubes, to which no precipitinogen had been added, the addition of organisms resulted in the formation of a typical firm disc. That the agglutinability of the organisms was unchanged after the treatment was shown by removing them from the mixture by centrifugation and resuspending them in saline, Type I and Type II sera. Agglutination occurred only in Type II serum.

The supernatant liquid from which the organisms were removed still contained bound antibody and free S. To show that the antibody had been bound by the free S, the material was diluted so that the serum was in a concentration (1:80) in which untreated serum would cause agglutination but would no longer give a precipitin reaction. To this mixture bacterial suspension was added but no agglutination occurred, nor had dilution of the mixture caused any precipitation.

The explanation of the results of Experiment 3 appears to be that in the presence of an excess of free S the entire mass of antibody combines with it to form a soluble product. Consequently little or no antibody combines with the S of the cell body. As a result, the bacteria are in an unaltered state of reactivity. That is, if an inhibition zone is produced by the free S in the type-specific precipitin reaction, the antibody is no longer capable of agglutinating type-specific organisms when they are introduced into the reaction mixture.

DISCUSSION

Heidelberger and Kendall (4) have suggested that the precipitin reaction follows certain rules which may be expressed in terms of the laws of mass action. In the study they employed the specific capsular polysaccharide of Type III Pneumococcus and an antibody solution derived from an homologous immune serum. Their results and interpretations are briefly as follows:

1. When the smallest amount of type-specific soluble substance (S) capable of producing a precipitate is added to antibody (A), the reaction takes the form

$$A + S \rightleftharpoons A\,S,$$

in which the ratio of antibody to S in the precipitate is about 120:1.

2. The precipitate in Equation 1 is capable of reacting with more S to a point at which both components are in equilibrium in solution and the composition of the precipitate is approximately 60:1.

$$A\,S + S \rightleftharpoons A\,S_2.$$

3. With the addition of slightly more S, the point of maximal precipitation is reached, and with further continued addition of S the precipitate gradually redissolves. This is assumed to be associated with the formation of a new combination of A and S which is soluble.

$$A\,S_2 + S \rightleftharpoons A\,S_3.$$

In the three equations the proportions of S combined with A vary as 1:2:3.

Although accurate quantitative estimations were not made, it can be seen that the reaction of type-specific agglutination follows, in general, the principles given by Heidelberger and Kendall for the precipitin reaction.

In Experiment 1 Equations 1 and 2 are realized. When bacteria, in an amount fractional of that required for complete absorption of the immune serum, are added to immune serum, a type-specific agglutination occurs. Substituting in Equation 1 of Heidelberger and Kendall, we derive

$$\text{I.}\quad A + S\ (\text{cellular}) \rightleftharpoons A\,S\ (\text{cellular}).$$

The antibody in the agglutinated mass is able to combine with additional S as shown by the fact that when more free S is added to the agglutinated material some of the S is bound. Heidelberger and Kendall have shown that this is not a simple adsorption. Hence,

$$\text{II.}\quad A\,S\ (\text{cellular}) + S\ (\text{free}) \rightleftharpoons A\,S_2\ (\text{cellular and free}).$$

In Experiment 2 the combined reagents may exist either in the form of A S or A S_2. In either case, the addition of sufficient free S causes the equilibrium to swing in the direction of Equation III. Under these conditions the antibody forms a soluble compound with S and the agglutinated organisms are redispersed to their original state of suspension.

III. (*a*) A S (cellular) + 2 S (free) $\rightleftarrows$ A S_3—agglutination redispersed

or

III. (*b*) A S_2 (cellular) + S (free) $\rightleftarrows$ A S_3—agglutination redispersed.

In this instance the antibody is dissociated from the cellular S or forms a soluble combination with the free S. But only a small amount of the cellular S is removed from the cells in this process for they can be agglutinated again in a normal manner with homologous immune serum.

In Experiment 3 the antibody is bound by free S to form A S_3, which is soluble. When organisms are subsequently added they are not agglutinated but, removed from the reaction mixture, they are still specifically agglutinable.

IV. A S_3 (free) + S (cellular) $\rightarrow$ no agglutination.

The zone phenomenon encountered in the present instances differs from that usually met with in agglutination reactions. In the agglutination reaction the prozone usually occurs in the presence of an excess of antibody. In the present experiments agglutination is inhibited by an excess of S, the relations usually present in the inhibition of precipitation.

It has previously been indicated that precipitation of a specific soluble antigen may take the form of agglutination in the presence of particulate matter. Nicolle (5) and Arkwright (6) demonstrated that by suspending particulate matter or heterologous bacteria in the soluble precipitinogen of *B. typhosus*, agglutination of the suspended material was produced by antityphoid serum. Jones (7) suspended collodion particles in solutions of antigens and then removed, by washing, all antigen but that adsorbed by the collodion. When the particulate matter was resuspended and added to serum of animals immunized to the antigen, agglutination occurred. The same reactions were obtained when bacteria were treated with non-bacterial antigens. Mudd and his associates (8) have recently confirmed Jones' results. White (9) has shown that saline extracts of organisms of the Salmonella group, or "carbohydrate-containing hap-

tens," prepared by treating the organisms with alkali, when added to homologous immune serum were able to inhibit the action of the somatic agglutinins for bacterial cells subsequently added to the mixture. No influence was exerted upon the flagellar agglutinins.

In the present experiments the same specifically reactive substance is present in both the precipitation and agglutination reactions. In the latter it is part of the bacterial cell; in the former it is chemically pure and separate from the cell. In both instances the reactions, whether agglutination or precipitation, appear to be controlled by the same laws.

SUMMARY

The experimental results which have been described demonstrate the following facts:

1. In the type-specific agglutination reaction, when the organisms are not present in sufficient numbers to absorb completely all the antibodies from the serum, more antibody is bound by cellular S than is required for the process of agglutination.

2. The excess of antibody thus bound can then unite with additional amounts of the specific substance when this is added in soluble form to the agglutinated material.

3. If an excess of the free S is added to an agglutinated mass of antibody and bacteria, the organisms are redispersed and in the suspended state are again specifically agglutinable.

4. When a solution of the specific polysaccharide is added in excess to an homologous immune serum, a prozone is created in which precipitation is inhibited; moreover, if, at this point, type-specific pneumococci are added to the mixture, inhibition of agglutination also occurs.

5. The reactive substance in the type-specific agglutination and precipitation reactions is the same, *i.e.*, the capsular polysaccharide. In the former instance it is combined in the bacterial cell; in the latter, it is in a soluble, chemically purified state and entirely separate from the body of the cell.

CONCLUSION

The results obtained in the agglutination reaction conform qualitatively with the principles described by Heidelberger and Kendall

for the phenomenon of precipitation. They indicate that the essential mechanism of the two reactions is identical and that the active reagents are the same in both.

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