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Separation and Properties of a Red Cell Sensitizing Substance from Streptococci

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ABSTRACT

MOSKOWITZ, MERWIN (Purdue University, Lafayette, Ind.). Separation and properties of a red cell sensitizing substance from streptococci. J. Bacteriol. **91**:2200–2204. 1966.—An antigen that binds onto red cells and causes them to be agglutinated by antiserum was separated from streptococci. Various procedures to extract the antigen from streptococci were investigated, and the greatest amount of antigen was obtained by extraction of cells with a phenol-water mixture. The reaction of the antigen with red cells was shown to be reversible by use of the Ashby mixed agglutination technique. The antigen also combines with a number of different tissues, and it was demonstrated that the antigen could be transferred from red cells to tissues and vice versa. An hypothesis is presented on the basis of these findings which suggests a possible role for this antigen in the etiology of rheumatic fever.

The agglutination of red cells by normal human sera or specific antisera after having been exposed to a culture filtrate or extracts of streptococci or staphylococci has been noted by many workers (3, 7, 9, 11, 13, 14, 16, 19) and was initially described in some detail by Pakula (14) and Rantz (15). These latter workers observed the antigen in a variety of gram-positive organisms, and Rantz called it a nonspecies specific substance. The behavior of this antigen was similar to the variety of other antigens that bind onto red cells and cause them to be agglutinated by antibodies against the antigen (for review, *see* 12). The antigen did not pass through cellophane dialysis tubing. It was stable at 100 C for 15 min up to *p*H 6, but was inactivated above *p*H 7 under these conditions (15). It was not related to the C polysaccharide (14). Its biological properties were not affected by trypsin, pepsin, or ribonuclease, and it appeared unlikely to be a protein (14).

The separation of the antigen from streptococci and staphylococci, the nature of its interaction with red cells, the binding of the antigen by tissues other than red cells, and the possible role of the antigen in the etiology of rheumatic fever have been under investigation in this laboratory for a number of years (1, 2, 4, 5, 6, 8, 10, 17; M. Moskowitz and B. Thompson, Bacteriol. Proc. p. 65, 1954; M. Moskowitz, Federation Proc. **14**:1578, 1955; M. Moskowitz and B. Thompson, Proc. Ind. Acad. Sci. **63**:60, 1954). Many of our earlier observations were subsequently described by the workers that have been cited, and our findings are in general agreement with theirs. The results of these investigations will be presented in this and following papers. In this paper, a method for extracting large quantities of the antigen from streptococci and some aspects of the nature of the interaction of antigen with cells and tissues are described.

MATERIALS AND METHODS

Buffered saline. Solution contained 1.42 g of Na_2HPO_4, 0.18 g of KH_2PO_4, and 8.418 g of NaCl, dissolved in water and made up to 1 liter. The *p*H was 7.3.

Assay of red cell sensitizing antigen. Serial twofold dilutions of the preparation were made with buffered saline in 0.2-ml volumes, and 0.2 ml of a washed 2% suspension of type O human red cells was added to each tube. After incubation at 37 C for 30 min, the tubes were centrifuged at 1,000 × *g* for 3 min, and the supernatant fluid was removed. The sensitized cells were washed three times with 1 ml of buffered saline and then made up to a volume of 0.4 ml with buffered saline. Two drops of this suspension was added to two drops of pooled human serum, and the mixture was incubated for 30 min at 37 C. The mixtures were centrifuged at 1,000 × *g* for 3 min, the pellet at the bottom gently dislodged, and the degree of agglutination observed macroscopically. The titer of the preparation was recorded as the reciprocal of the last dilution in which the definite clumps were present with no evidence of free cells, and this is referred to as the sensitizing activity of the preparation.

Titration of antiserum. Serum was obtained from

the blood bank from presumably normal individuals and then pooled. It was kept frozen at −18 C until just before use. Serial twofold dilutions of the sera were made in 1-ml quantities. A volume (0.1 ml) of these dilutions was added to serological tubes (10 by 75 mm), and 0.1 ml of a 1% suspension of red cells sensitized as described above was added to the serum dilutions. After standing at 37 C for 30 min, the tubes were centrifuged at 1,000 × *g* for 2 min and the agglutination was determined as described above.

Preparation of streptococcal cells. The cells were cultured in Brain Heart Infusion (Difco) broth (37 g in 800 ml of distilled water) to which was added 100 ml of a buffered glucose solution (prepared by adding 200 g of glucose, 10 g of KH_2PO_4, and 10 g of K_2HPO_4 to 780 ml of distilled water), and 100 ml of a buffered salt solution (NaCl, 30 g; Na_2CO_3, 25 g; Na_2HPO_4, 15 g; and 1 liter of water). Each solution was autoclaved separately at 15 psi for 15 min and combined immediately prior to inoculation. Final *p*H was 7.2. One-liter quantities of this medium were distributed in 2-liter Erlenmeyer flasks. Amounts of 10 ml of a 12-hr culture of group A *Streptococcus pyogenes*, strain 1-RP41 (obtained from Rebecca Lancefield of the Rockefeller Institute), were inoculated into each flask. (The cultures used for inoculating the flasks were subcultured a minimum of four times at 12-hr intervals prior to inoculation.) The flasks were incubated at 37 C for 18 hr and then inactivated at 60 C for 2 hr. The inactivated cultures were centrifuged on a Sharples centrifuge or on a Servall continuous-flow centrifuge. The pellet was suspended in a 0.23 M NaCl solution in a concentration of approximately 5%, recentrifuged, and then washed successively in a similar concentration in 0.05 N HCl, 0.01 N HCl, and twice with distilled water. Immediately after washing, water was added to the cell pellet to make a smooth paste, and the cells were lyophilized. The cells were stored at −18 C until used.

RESULTS

Extraction of the antigen from S. pyogenes. In previous experiments, it was observed that culture filtrates had sensitizing activities of 32 to 64. Water extraction of a 1% suspension of cells at 25 C and 100 C for 1 hr usually yeilded extracts with a sensitizing activity of 100 to 200; however, extracts with greater sensitizing activity, some as high as 5,000, were occasionally obtained. Formamide extraction, acid extraction by use of Lancefield's procedure, and extraction with 95% phenol yielded extracts with a low sensitizing activity, never greater than 50. These results are similar to those which others have obtained by use of these procedures (9, 14, 15).

The extraction of a 1% suspension of cells in water with an equal volume of a 95% aqueous phenol solution yielded an extract with a sensitizing activity of 40,000. A 1% suspension of lyophilized cells was made in distilled water by adding very small amounts of water to the cells, mixing with a rubber policeman to form a paste, and progressively adding small amounts of water and mixing until the 1% suspension was attained. An equal volume of a 95% aqueous reagent phenol solution was added to the cell suspension, the mixture was gently shaken, and the *p*H was adjusted to 4.7 with 1 M NaOH solution. The mixture was centrifuged at about 1,000 × *g* for 20 min, and the aqueous phase was removed with a pipette. Some cells were often present at the phenol-water interphase, and care was taken not to remove any of the cells. The water layer was placed in cellophane tubing and dialyzed against three changes of 80 volumes of water at 5 C. After dialysis, the extract was centrifuged to remove any precipitate that formed and was stored at −18 C until used.

Variations in this procedure had an effect on the amount of antigen extracted and on the composition of the extract. At the *p*H specified in the procedure, a maximal yield of antigen was obtained per unit weight of the extract. When the *p*H of the mixture was increased above 4.7, the amount of antigen obtained remained the same, but the amount of material that was removed from the cells was increased. Thus, at *p*H 6.5, the weight of the extract was as much as twice that obtained at *p*H 4.7. The extract obtained at *p*H 4.7 showed no absorption peak at 2,600 A, but there was an increase in absorbance at this wavelength as a function of increasing *p*H. If the *p*H was decreased below 4.7, the yield was decreased, and, at *p*H 3, no antigen was obtained.

The manner in which the streptococci were harvested and handled also influences the amount of material released from the cells. If the cells were exposed to much mechanical agitation after being removed from the medium, if there were variations in the washing procedures, or if the cells were frozen and thawed, larger amounts of inactive materials were released from the cells even at *p*H 4.7.

Absorption of the antigen to bacterial cells. The *p*H of a 1% suspension of cells in the phenol-water mixture at *p*H 4.7 was lowered by the step-wise addition of 1 M HCl. At various *p*H intervals, samples were removed from the mixture, the cells were removed by centrifugation, and the extract was tested for sensitizing activity. With decrease in *p*H, less sensitizing activity was found in the extracts, and no activity was observed at *p*H 3 (Fig. 1). The *p*H of the mixture was raised to 4.7 with 1 M NaOH, and the sensitizing activity of the extract was the same as at the start of the experiment. The cells that were removed at the various *p*H intervals were resuspended in a phenol-water mixture, and the *p*H was adjusted to 4.7. The sensitizing activity of

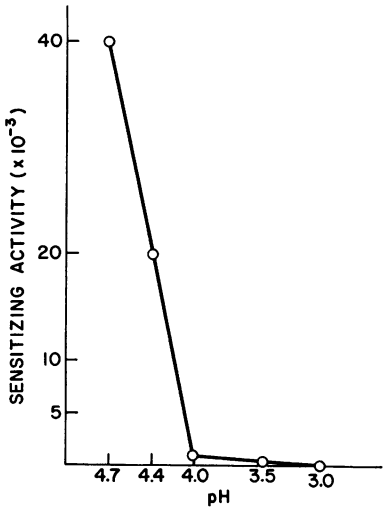

FIG. 1. *Decrease in sensitizing activity of phenol-water extraction mixture with decrease in pH.*

these extracts was equal to the activity that had been removed from the original mixture at the respective *p*H.

A 1% suspension of cells was made in water; the resultant *p*H was 5. The sensitizing activity of the water phase was 100. The *p*H of the suspension was decreased to 3, and, at this *p*H, no sensitizing activity was detected in the water phase. The *p*H of the suspension was then raised to 5, and the sensitizing activity in the water phase was 100. The *p*H of the suspension was increased to 7, and the sensitizing activity in the water phase was 1,000. Further increases of the *p*H to 9 did not result in a further increase in the amount of sensitizing activity present in the water phase. Reduction of the *p*H to 5 resulted in a decrease of the sensitizing activity in the water phase to 100.

The decrease in the amount of sensitizing activity present in the suspension as the *p*H was lowered and the increase as the *p*H was raised was invariably observed with the phenol-water mixture; however, variations were observed in water suspension prepared from different batches of cells. Although all suspensions had a sensitizing activity of about 100 at *p*H 5, and the sensitizing activity in the water phase decreased as a function of decreased *p*H, the sensitizing activity would occasionally be as high as 10,000 when the *p*H was raised to 7.

Relationship of agglutinin titer of serum to degree of sensitization of red cells. The maximal serum agglutinin titer was obtained with red cells sensitized with an extract having a sensitizing activity of 128 or greater. If the red cells were sensitized with an extract with an activity less than 128, there was a proportional decrease in the agglutinin titer of the serum. Cells sensitized with an extract which has a titer of 128 are referred to as "maximally sensitized." The decrease in agglutinin titer with red cells sensitized with extracts which have an activity less than 128 is undoubtedly related to the amount of antigen bound to the sensitized cells, and this relationship thus provides a measure of variations in the amount of antigen bound to red cells that may occur under different conditions.

Elution of the antigens from sensitized red cells. Red cells were maximally sensitized with antigen and were washed five times with buffered saline in a 20% concentration. No antigen was detected in the final three washings. The cells were suspended in buffered saline in a 20% concentration, allowed to stand at 37 C for 1 hr, and centrifuged; the supernatant fluids were tested for the presence of antigen. In three of six experiments, the eluates sensitized red cells. The amount of antigen recovered in the supernatant fluid was apparently very small, inasmuch as the red cells sensitized by this fluid were barely agglutinated by the serum. The fact that only small amounts of antigen were released suggested the possibility that the reaction of the antigen with red cells was reversible, and, as soon as some is released from a red cell, it reattaches to the same or a different red cell.

Transfer of antigen from sensitized to normal red cells. In an attempt to test the reversibility of the binding of the antigen to red cells, an experiment patterned after the Ashby mixed agglutination technique was carried out. One volume of a 1% suspension of maximally sensitized cells was added to 1 volume of a 1% suspension of normal cells in a tube. The mixture of cells was placed at 37 C and samples of the mixture were removed immediately and at 10-min intervals thereafter over a period of 2 hr. One volume of the mixture of cells that was removed was added to 1 volume of serum in a tube, incubated at 37 C for 30 min, and then gently poured onto a slide. The mixture was scored microscopically for the number of cells agglutinated and degree of agglutination. Immediately after mixing, approximately 50% of the cells were free, and the remaining 50% were agglutinated in large clumps. This result would be expected if only 50% of the cells in the mixture were sensitized. After 10- and 20-min intervals, the relative proportion of free cells decreased. After 30 min, there were no free cells, and mixtures of relatively large clumps and relatively small clumps were detected. In the samples removed after 1 hr, there was less varia-

bility in the size of the clumps, and, at the end of 2 hr, the clumps were similar in size.

Binding of antigen to tissues. The ability of the antigen to combine with various tissues was tested in the following manner: pieces of liver, spleen, heart muscle, heart valves, and aorta were removed from an adult human at autopsy. The tissues were minced with scissors and washed with buffered saline. The minced tissues were suspended to a concentration of 5% in buffered saline and mixed with an equal volume of antigen preparation with an activity of 128. After incubation for 30 min at 37 C, the mixture was centrifuged and the amount of antigen remaining in the supernatant fluid was titrated. In all cases, the sensitizing activity of the supernatant fluid was 2 or 4, indicating that the tissues had bound the antigen. A study of the binding of antigen with tissues from different individuals demonstrated that the ability to bind the antigen varies from individual to individual. The details of these experiments will be published later.

Transfer of antigen from tissue to red cells and from red cells to tissue. The transfer of antigen to red cells from tissue and vice versa was determined by use of the modified Ashby technique. Minced heart tissue was mixed with antigen as described above and washed five times with buffered saline. No antigen was detected in the last three washings. One volume of this tissue suspension was mixed with 1 volume of a 2% red cell suspension, and the mixture was incubated at 37 C. At different time intervals, portions of the mixture were removed, the red cells were separated from the tissue fragments by permitting the latter to settle to the bottom of the tube, and the remaining red cell suspension removed. The cells were washed and tested for their agglutinability by serum. The red cells removed immediately after mixing with the sensitized tissue fragments were not agglutinated. After 15 min, clumps of two and three cells were observed, and, at 30 min and 1 hr, the degree of clumping increased. At the end of 2 hr, large agglutinates were observed. The test was also carried out with tissues that had not been mixed with antigen, and, in this case, no agglutination was observed.

In an attempt to determine whether antigen could be passed from red cells to tissue, an experiment identical to that carried out above was set up, except that the cells were maximally sensitized with antigen and the tissue fragments were not. The serum agglutinin titer with these cells was 64 at the start of the experiment and decreased to 1 over the experimental period of 2 hr. The serum agglutinin titer remained unchanged when determined with sensitized red cells that were handled in the same manner but without tissue added to the tubes. The decrease in the agglutinating ability of the red cells suggests that there was less antigen present in them and that the antigen had been transferred from red cells to the tissue. To test whether this was the case, the tissue fragments remaining in this experiment were washed with buffered saline and mixed with normal red cells in an experiment identical with that in the above paragraph. After 2 hr of incubation, the cells were strongly agglutinated with serum.

DISCUSSION

The observation that, after extraction with the phenol-water mixture at *p*H 4.7, the antigen binds onto the bacterial cell as the *p*H is lowered, suggests that the antigen is attached to the cell by an ionic bond. However, inasmuch as the antigen is not readily released from the bacterial cells in water alone at *p*H 5, it may exist below the surface of the cell or is associated with another compound on the surface of the cell by other than ionic binding. If the latter situation obtains, the large amount of antigen released into the phenol-water mixture may be due to the phenol disrupting the cell surface, or breaking the postulated association of the antigen with another compound, causing it to be released into the water. After it is present in the water phase, and the *p*H is lowered, it may combine with charged sites on the surface of the bacterial cell.

The conditions which are described for preparing bacterial cells, under which minimal amounts of extraneous material are extracted with the phenol-water mixture, were arrived at after trying a variety of methods for preparing the cells. Seemingly slight variations in this procedure, such as washing the cells at a slightly higher *p*H than is described, caused more materials to be released from the cell in addition to the antigen. Such variations also caused more materials to be released from the cells in water alone. When more material was released into water, however, there usually was an increase in the amount of antigen released compared with that released when the cells are prepared in the manner described. The variation in the amount of antigen released in water is probably related to the condition of the cell surface, and, if a particular manner of handling the cells has a more disrupting effect on the organization of the cell surface, it may cause the release of antigen in a manner similar to that postulated to be due to the action of phenol. The larger amount of antigen released into water when the *p*H is raised from 5 to 7 may also be due to the effect of the higher *p*H on the cell surface.

The antigen can bind onto a variety of tissues,

in addition to red cells. This has also been observed by Stewart (18). Apparently there are receptors for the antigen on cells of these tissues. The experiments with use of the modified Ashby technique indicate that the binding of the antigen to the receptors on the red cells and tissues is reversible. The antigen can pass from a sensitized red cell to a normal red cell, antigen from red cells can be transferred to tissues, and tissue-bound antigen can be transferred to red cells.

Rheumatic fever occurs after infection with *S. pyogenes*, and, although this antigen is present in a variety of gram-positive organisms, it appears possible that it could play a role in the pathogenesis of rheumatic fever. If the antigen was concentrated at various tissue sites, and an antigen-antibody reaction occurred while the antigen is attached to a tissue, damage may result to that tissue.

The nature of infections with *S. pyogenes* may result in the release of larger amounts of the antigen into the circulation than when infections occur with other gram-positive organisms, and thus larger amounts could be bound to tissues. Another factor that could result in the release of larger amounts of antigen from *S. pyogenes* is that relatively larger amounts may be released from it under physiological conditions. It has been observed that under a number of different conditions, much greater amounts of antigen are released from *S. pyogenes* than from other gram-positive organisms (5).

The ability of the antigen to pass from red cells to tissues and vice versa indicates a mechanism whereby the antigen can be distributed to tissues in the body. If the antigen is released at the focus of a streptococcal infection and enters the blood, it can combine with red cells and then be transferred to various tissues. The amount of antigen that remains bound to the red cells and tissues probably depends upon the number of receptors on red cells and the cells of a particular tissue, and on possible differences in the dissociation constant of the antigen with the receptors at the different locations. If a given tissue has relatively more receptors than red cells, and there is a lower dissociation constant of the antigen with the receptor on the tissue than on the red cell, the antigen would be concentrated at that site.

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LITERATURE CITED

1. CASEY, H. 1958. The relationship of the red cell sensitizing antigen of group A streptococci to their cell walls. Ph.D. Thesis, Purdue Univ., Lafayette, Ind.
2. COHEN, P. 1964. Immunological studies of red cell sensitizing substances in gram-positive organisms. Ph.D. Thesis, Purdue Univ., Lafayette, Ind.
3. FRASER, K. B. 1954. Antibody in glandular-fever sera to an antigen common to streptococci and staphylococci. J. Pathol. Bacteriol. **67**:301–309.
4. FUCILLO, D. A. 1962. Location of streptococcal antigen in tissues. Ph.D. Thesis, Purdue Univ., Lafayette, Ind.
5. HANKINS, W. A. 1959. Assay of an antigen common to several bacterial species. M.S. Thesis, Purdue Univ., Lafayette, Ind.
6. HANKINS, W. A. 1963. The relationship of a red cell sensitizing antigen to the bacterial cell. Ph.D. Thesis, Purdue Univ., Lafayette, Ind.
7. HARRIS, T. N., AND S. HARRIS. 1953. Agglutination by human sera of erythrocytes incubated with streptococcal culture concentrates. J. Bacteriol. **66**:159–165.
8. JACKSON, R. W., 1963. Studies on the nature of a red cell sensitizing substance from streptococci. Ph.D. Thesis, Purdue Univ., Lafayette, Ind.
9. KIRBY, W. M. M. 1951. Hemagglutination reaction in streptococcal infections and acute rheumatic fever. Proc. Soc. Exptl. Biol. Med. **78**:519–524.
10. MORALES, H. 1957. Studies on the tissue receptors for the red cell altering factor of streptococci. M.S. Thesis, Purdue Univ., Lafayette, Ind.
11. MOSKOWITZ, M., AND H. P. TREFFERS. 1950. An agglutinin in normal sera for periodate-treated red cells. Science **3**:717.
12. NETER, E. 1956. Bacterial hemagglutination. Bacteriol Rev. **20**:166.
13. NETER, E., AND E. A. GORZYNSKI. 1959. Studies on indirect staphylococcal hemagglutination. Z. Immunitaetsforsch. **118**:269–288.
14. PAKULA, R. 1952. Polysaccharides in hemolytic streptococci, analysis by methods of hemagglutination and precipitation. Med. Doswiadezalna Mikrobiol. **4**:197–216.
15. RANTZ, L. A., A. ZUCKERMAN, AND E. RANDALL. 1952. Hemolysis of red blood cells treated by bacterial filtrates in the presence of serum and complement. J. Lab. Clin. Med. **39**:443–448.
16. ROUNTREE, P. M., AND R. G. H. BARBOUR. 1952. Antibody to the erythrocyte-coating polysaccharide of staphylococci: its occurrence in human sera. Australasian Ann. Med. **1**:80.
17. SONNENWIRTH, A. C. 1953. Studies on the inhibitor for the red cell altering factor of streptococci. M.S. Thesis, Purdue Univ., Lafayette, Ind.
18. STEWART, F. S., AND W. T. MARTIN. 1962. Adsorption of a streptococcal red cell sensitizing antigen to various tissues. J. Pathol. Bacteriol. **84**:251–253.
19. STEWART, F. S., T. W. STEELE, AND W. T. MARTIN. 1959. Mechanisms involved in the production of red cell panagglutinability by streptococcal cultures. Immunology **2**:285–294.