AGGLUTINATION BY HUMAN SERA OF ERYTHROCYTES INCUBATED WITH STREPTOCOCCAL CULTURE CONCENTRATES[1]

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It was demonstrated by Keogh *et al.* (1947) and Warburton *et al.* (1949) that several products of bacterial cultures could be adsorbed to erythrocytes, which then could be agglutinated by sera containing antibodies to those bacterial antigens. This approach was developed in the case of a constituent of tuberculin by Middlebrook and Dubos (1948) and Rothbard *et al.* (1950). The work of the latter groups served to emphasize the point that a single constituent might be adsorbed to the erythrocyte from a mixture of antigens by a selective adsorption. Recently this technique has been applied to work with the plague bacillus (Amies, 1951), the hemolytic streptococcus (Kirby, 1951), and the colon bacillus (Neter *et al.*, 1952).

The present study arose in the course of an exploration of antigens of the hemolytic streptococcus in terms of a possible agent in the pathogenesis of rheumatic fever. The current emphasis in this work is on the extracellular antigens of the hemolytic streptococcus, as found in concentrates of streptococcal culture supernates. Since there are presumably a number of streptococcal products in such concentrates, it was decided to undertake the present study to see whether erythrocytes would adsorb any constituent of the culture supernate concentrates to which antibodies might be present in human sera. Concurrent studies on the entire culture supernate concentrates are in progress.

MATERIALS AND METHODS

Streptococcal preparations. Hemolytic streptococci of a number of strains were cultivated in 12 liter volumes in a dialyzate medium described elsewhere (Harris and Friedman, 1949) and in the synthetic medium developed by Bernheimer *et al.* (1942). After incubation for 18 hours at 37 C the cultures were freed of organisms by centrifugation and concentrated by pervaporation, dialysis, and lyophilization as described elsewhere (Harris and Friedman, 1949). These preparations were dissolved in the indicated ranges of concentration for adsorption experiments. Control batches of uninoculated media of both types were processed to the lyophilized state in the same manner.

The greater part of the work described below was done with the H44 strain of type 4, group A, β-hemolytic streptococci which has been used in earlier work reported from this laboratory. Other strains, involving a number of other types within group A, also were used in these studies. The latter were from a collection originally made available through the courtesy of Dr. Rebecca C. Lancefield.

Sheep erythrocytes. These were obtained from lots of blood kindly supplied by Sharp and Dohme, Inc. Two lots were obtained each week, involving bleedings from two sheep of a group of animals bled in rotation. The designations of the blood specimens provided an indication of the animal used. The sheep erythrocytes were collected by centrifugation, washed twice in buffered saline solution, and then made up to a 40 per cent suspension for immediate use.

Sera. Sera of patients with rheumatic fever in various stages of clinical activity were available in our collection of such sera, drawn from patients at The Children's Hospital of Philadelphia, The Philadelphia General Hospital, The Hospital of the University of Pennsylvania, and The Children's Seashore House for Invalid Children at Atlantic City. Sera from patients with scarlet fever were obtained at The Philadelphia Hospital for Contagious Diseases, through the courtesy of Dr. A. C. LaBoccetta. These were drawn from patients in the first half week of their disease and in the second half of the third week, just before

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discharge from the hospital. The pairs of sera drawn from individual patients were tested, in each case, within a given experiment.

Sera were maintained in the frozen state. For use in the tests to be described below they were thawed, placed in a water bath at 56 C for 30 minutes, and absorbed twice with one-tenth volume of packed sheep erythrocytes.

Conduct of the test. In testing a streptococcal preparation for its activity in the erythrocyte adsorption-agglutination test, or a serum for its agglutination titer in such a test, the following procedure generally was employed: Dilutions of the streptococcal concentrate were made, in the desired range, in a given volume of buffered isotonic saline solution. One-eighth volume of a 40 per cent suspension of washed sheep erythrocytes was added then to each dilution of the streptococcal concentrate, and incubation was carried out at the times and temperatures indicated below. Then the cells were washed three times and made up to a final concentration of 0.25 per cent.

Serial twofold dilutions of sera were prepared in volumes of 0.4 ml. To these dilutions were added 0.2 ml volumes of the sheep erythrocyte suspension. The tubes were shaken vigorously and incubated for 30 minutes at room temperature and then overnight at 4 C. Tests were read after shaking the tubes again. Partial or complete agglutination was demonstrable clearly at this concentration of erythrocytes.

RESULTS

The hemagglutination reaction. In early experiments the hemagglutination reaction was adapted directly from Middlebrook and Dubos (1948) to the supernates of streptococcal cultures concentrated as described above. In these experiments it was found that a positive hemagglutination test could be elicited by the interaction of certain human sera with erythrocytes sensitized by a given range of concentration of the streptococcal preparations. As the concentration of the streptococcal preparation used for sensitization was decreased below this range, the apparent hemagglutinating titer of the serum fell. As the concentration of the sensitizing streptococcal preparation was increased, a point was reached often at which the concomitant increase in concentration of the streptococcal hemolysin interfered with the hemagglutination reaction by causing hemolysis of the erythrocytes. Typical data from this initial series of experiments are shown in table 1.

Variations in the technique of the test. Early experiments involving this reaction were performed in order to determine optimal conditions for the sensitization of the erythrocytes and for the agglutination of the sensitized cells. The sensitization of the cells at 37 C was found to take place within 15 minutes, to the extent that such adsorption would take place in an hour, as can be seen in table 2. In other experiments this interval was extended to 2 hours with similar results. At room temperature the adsorption took place more slowly and was slightly less than maximal in half an hour.

TABLE 1

Agglutination by normal human serum of sheep erythrocytes sensitized by concentrates of streptococcal culture filtrates

STREPTOCOCCAL CONCENTRATE		SERUM DILUTION				
		1:4	1:8	1:16	1:32	1:64
CS 48 (SM)	1%	h	h	h	h	h
	0.5%	2	2	2	—	—
	0.25%	2	2	2	—	—
	0.125%	2	2	2	—	—
	0.06%	2	2	—	—	—
	0.03%	2	—	—	—	—
CS 48 (DM)	1%	h	h	h	h	h
	0.5%	2	2	2	—	—
	0.25%	2	2	—	—	—
	0.125%	2	—	—	—	—

2—strong agglutination; h—hemolyzed; SM—synthetic medium; DM—dialyzate medium.

The time required for the agglutination of the sensitized cells also was studied in order to determine the minimum time required for the test. It was found that maximal agglutination under these circumstances occurred within a half hour and that incubation at room temperature was as effective as incubation at 37 C. The incubation at room temperature was followed by overnight incubation in the refrigerator to allow the cells to settle before the degree of agglutination was read. Alternatively the tubes could be subjected to centrifugation, with similar results, at 500 rpm for 2 minutes after the half hour at room temperature.

Experiments with tannic acid. It has been

shown by Boyden (1951) that previous treatment of erythrocytes with a solution of tannic acid enhances their adsorption of substances in solution, as indicated by subsequent hemagglutination tests. In an attempt to apply this observation to the system under study, sheep erythrocytes were treated with a solution of tannic acid (1:20,000), then washed and treated in the usual manner with concentrates of streptococcal culture filtrates. On subsequent hemagglutination tests of such cells with known positive sera it was not found, however, that the apparent hemagglutinin titers of the sera were increased, but rather that no agglutination reaction was obtained at all.

Variability of erythrocytes from different sheep as adsorbents. In the course of early experiments variations were encountered among the results obtained with different batches of sheep erythrocytes. It soon became apparent that these variations were associated with the sheep from which the blood had been obtained, and further tests confirmed this. It was found that of 6 sheep tested the erythrocytes of 3 gave optimal reactions in this test. The erythrocytes of two other sheep, when used in this test, resulted in a reduction of the apparent titer of a standard sera by one twofold step of dilution with smaller cell aggregates. The erythrocytes of one sheep gave no positive test at all within the threshold of sensitivity of this test. It was found that suspensions prepared from different bleedings of a given animal, when adsorbed with a given dilution of an antigen preparation, would be agglutinated by a standard serum to the same titer.

It was found also that no apparent relationship existed between the effectiveness of cells of various sheep in the hemagglutination tests and their effectiveness in adsorbing the normally occurring antisheep erythrocyte agglutinins from sera in preparation for the test.

In an effort to obviate the necessity for adsorbing out agglutinins for sheep erythrocytes from human sera in preparation for the use of such sera in this hemagglutination test, attempts were made to use human erythrocytes of type O blood. It was found, however, that of the several specimens of human blood tested none could be agglutinated by known positive sera after incubation with standard preparations of antigen.

The streptococcal concentrates. Hemolytic effects. As indicated above, there were many concentrates of streptococcal culture filtrates in which the procedure of the adsorption test resulted in hemolysis of the cells, presumably because of the presence of sufficiently high concentrations of the O-hemolysin in those preparations. Bubbling oxygen through the solution failed to obviate this difficulty, but incubation with cholesterol (at 2 mg per ml, for 10 minutes at room temperature, in a rotating device) did so. The cholesterol was removed by filtration, and similar treatment of nonhemolytic preparations of culture concentrates did not indicate any loss of the substance involved in the agglutination test.

TABLE 2

Effect of variations of time and temperature on the sensitization of sheep erythrocytes by streptococcal culture filtrate

TIME OF SENSITIZATION	CONC OF STREPTOCOCCAL FILTRATE	SENSITIZATION AT 37 C				SENSITIZATION AT ROOM TEMPERATURE			
		Serum dilution				Serum dilution			
		8	16	32	64	8	16	32	64
min	%								
60	0.25	2	2	2	—	2	2	2	—
	0.12	2	2	—	—	2	2	—	—
	0.06	2	1	—	—	2	1	—	—
30	0.25	2	2	2	—	2	2	1	—
	0.12	2	2	—	—	2	2	—	—
	0.06	2	—	—	—	1	—	—	—
15	0.25	2	2	2	—	2	2	—	—
	0.12	2	2	—	—	2	—	—	—
	0.06	2	1	—	—	1	—	—	—

2—strong agglutination; 1—weak agglutination.

The occurrence of the erythrocyte sensitizing substance in filtrates of various strains of hemolytic streptococci. The initial experiments in this study were carried out with two preparations of lyophilized concentrates of streptococcal culture filtrates, both derived from cultures of the H44 strain, one on dialyzate and one on synthetic medium. Subsequently, experiments were done to determine the relative concentration of the erythrocyte sensitizing substance in concentrates of other cultures of the same strain and of cultures of other strains. It was found that concentrates of filtrates contained this substance in the case

of the majority of strains tested, and that variations occurred among preparations derived from a given strain in terms of the concentration of red cell adsorbed substance present. Typical data, for a number of the dialyzate-medium preparations tested, are shown in table 3. The concentration of each preparation is the same, relative to dry weight, in order to allow expression of the differences in concentration of red cell adsorbed substance in various preparations. As control material, concentrates of uninoculated culture media are included.

TABLE 3

Sensitization of sheep erythrocytes by typical culture filtrates of various strains of hemolytic streptococci (dialyzate medium)

CULTURE NUMBER	TYPE OR STRAIN	AGGLUTINATION TEST, SERUM DILUTION			
		1:8	1:16	1:32	1:64
48	H44	2	1	—	—
40A	H44	2	2	1	—
49	H44	1	—	—	—
46A	C203	2	2	1	—
46B	C203	2	—	—	—
45	C203	2	1	—	—
5	NY5	2	1	—	—
39	T25	2	2	1	—
32	T25	2	1	—	—
40C	T40	2	1	—	—
Concentrate of medium		—	—	—	—

Distinction of the red blood cell adsorbed substance from the complement-fixing antigen(s) of hemolytic streptococcal culture supernates. During the course of these experiments there have been studies in this laboratory of another streptococcal antigen, or group of antigens, identified in concentrates of streptococcal culture supernates (and therefore designated CSA) by complement-fixation with certain human sera. It was considered important to ascertain whether red cell adsorbed substance and CSA might be the same or different substances for two reasons: first, because both are found and measured in the same source materials, and, second, because these are the only two extracellular antigens of the hemolytic streptococcus which are measured by aggregation or complement-fixation.

Fractionation of culture filtrate concentrates with ammonium sulfate. In a first approach to the question of identity or difference of the CSA and red cell adsorbed substance antigens, a lyophilized culture filtrate concentrate showing both activities was dissolved at a concentration corresponding to a sufficiently high titer with respect to each activity. This solution was introduced into a dialysis bag and dialyzed with continuous rocking against one volume of a saturated solution of ammonium sulfate, at pH 7, for 24 hours at 4 C. The contents of the bag then were transferred to a tube for centrifugation. The resultant sediment was designated as the 0 to 50 per cent saturated fraction, and the clear supernate was measured, placed in a bag, and dialyzed against a saturated solution of ammonium sulfate, as before. In this manner several fractions were obtained, as indicated in table 4, and these fractions were tested for activity as CSA and red cell adsorbed substance.

In three such experiments it was found that complement-fixing activity was heavily concentrated in the 0 to 50 per cent saturated fraction, with successively diminishing traces in the 50 to 75 per cent fraction and the 75 to 100 per cent fraction. The red cell adsorbed substance activity, on the other hand, appeared in lower degree than the original in the first two fractions and then rose in the 75 to 100 per cent saturated fraction. The data obtained in a typical experiment of this series are shown in table 4.

Removal of red cell adsorbed substance from streptococcal culture filtrates by adsorption on concentrated suspensions of sheep erythrocytes. In another series of experiments directed at the possible relationships of red cell adsorbed substance and CSA, use was made of the assumption that the red cell adsorbed substance antigen is adsorbed to the sheep erythrocyte in the course of sensitization. Portions of a suspension of washed red blood cells were placed in a series of pointed centrifuge tubes so that on centrifugation and removal of the supernate 0.4 ml of packed erythrocytes remained in each tube. Six ml of an appropriate concentration of a culture filtrate concentrate then were placed in the first tube, which was incubated for 1 hour at 37 C with frequent shaking. After the incubation the cells were sedimented by centrifugation and the supernate was withdrawn. An 0.8 ml portion was removed for subsequent tests, and the remainder of the supernate was added to another of the tubes containing packed erythrocytes. This procedure was re-

peated several times, and the portions removed after each adsorption were tested simultaneously for CSA and red cell adsorbed substance activity. It was found, as can be seen in table 5, that successive adsorption by packed erythrocytes caused progressive reduction in the activity of the solution in the adsorption-agglutination test but resulted in no measurable difference in complement-fixing activity.

Comparative titers of given sera against red cell adsorbed substance and CSA. As a final approach to the question of a possible relationship between red cell adsorbed substance and CSA, the titers of a number of sera were determined in the adsorption-agglutination test described here and in the complement-fixation test by which CSA is identified. The relative titers of given sera in the two tests varied from one extreme, in which the antired cell adsorbed substance titer of the serum was 16 times the anti-CSA titer, to the opposite, in which sera each showed an anti-CSA titer 12 times its antired cell adsorbed substance titer. Between these extremes was an irregular distribution of titer differences.

TABLE 4

Effect of fractionation with ammonium sulfate on concentration of red cell sensitizing factor and complement-fixing antigens of streptococcal culture filtrates

STREPTOCOCCAL FILTRATE	COMPLEMENT FIXATION							AGGLUTINATION TEST*			
	Antigen, mg/ml							Serum dilution			
	1:8	1:16	1:32	1:64	1:28	1:256	1:512	1:8	1:16	1:32	1:64
Original	0	0	c	c	c	c	c	2	2	2	—
Fractions:											
0–50% saturation	0	0	0	0	0	w	c	2	1	—	—
50–75% saturation	0	w	c	c	c	c	c	2	1	—	—
75–100% saturation	c	c	c	c	c	c	c	2	2	2	—

* Test performed with cells sensitized in solutions of 0.12 mg per ml of the respective fractions.

Application to the study of sera obtained from patients with rheumatic fever and scarlet fever. Rheumatic fever. The adsorption-agglutination test described above was applied to the examination of sera from 90 presumably normal children and 50 with rheumatic fever. Of the latter, the disease was considered to be in an active stage in 30 cases, subsiding in 10, and inactive in 10. Of the 90 normal sera 20 showed a titer of less than 6, the remainder being distributed in a broad frequency distribution from 6 to 256, with a mode of 32 and a geometric mean titer of 46. The 30 serum specimens representing active rheumatic fever were distributed from less than 6 to 128, with a geometric mean titer of 48. The specimens from patients with rheumatic fever in a state of subsiding activity showed titers from less than 6 to 64, with a geometric mean titer of 26; and those representing inactive rheumatic fever ranged from a titer of 6 to one of 192, with a geometric mean titer of 32. Thus, there was no marked difference between the distribution of antired cell adsorbed substance titers in normals and in active rheumatics, nor was any correlation observed between clinical activity of the rheumatic process and range or mean of titers.

Scarlet fever. A considerable number of sera were available from patients with an acute streptococcal infection, scarlet fever. These sera were paired, each patient having one specimen drawn within the first half week of the disease and the other one toward the end of the third week of the infection. These sera were examined for antired cell adsorbed substance titer, both members of each pair being included within the same test in each instance. A total of 89 such pairs was examined, and in the great majority of these, 79 or 89 per cent, the antired cell adsorbed substance titer in the convalescent stage of the disease was considered to show no significant difference from its titer at the beginning (less than two steps of twofold dilution of serum). In 5 pairs of sera the second specimen showed a titer four times as high as the first serum, and in 3 pairs the difference was eightfold or more. In one pair there was a fall in titer from the acute to the convalescent stage of the disease, the latter

titer being a quarter of the former. The patients whose antired cell adsorbed substance titer had shown any rise at all from the acute to the convalescent stage of scarlet fever were traced, with the aid of the Visiting Nurse Society, and questioned as to the occurrence of any complications or sequelae of the scarlet fever, but no such developments were discovered.

TABLE 5

Treatment of streptococcal culture filtrate with successive portions of packed sheep red blood cells. Effect on concentrations of red cell sensitizing factor and of complement-fixing antigens.

NUMBER OF ABSORPTIONS WITH RED BLOOD CELLS	COMPLEMENT FIXATION					AGGLUTINATION TEST				
	Antigen tested at					Serum dilution				
	1:2	1:4	1:8	1:16	1:32	1:8	1:16	1:32	1:64	1:128
0	0	0	tr	w	c	2	2	2	2	1
1	0	0	0	w	c	2	2	2	2	1
2	0	0	0	w	c	2	2	2	1	—
3	0	0	0	w	c	2	2	1	—	—
4	0	0	tr	w	c	2	2	—	—	—
5	0	0	tr	w	c	—	—	—	—	—

0—no hemolysis; tr—trace of hemolysis; w—weak hemolysis; c—complete hemolysis.

DISCUSSION

Technical aspects of the adsorption. A considerable number of bacterial substances thus far have been shown to react with or be adsorbed to the surface of erythrocytes in such a manner as to make possible agglutination of these red blood cells by antibody specific to the adsorbed substance. The differences observed among the technical aspects of the reaction may indicate that different structures in the erythrocyte wall or bacterial substances of substantially different molecular species might be involved in these reactions. Thus, the time allowed for adsorption has been as little as five to ten minutes at room temperature in the case of *Hemophilus pertussis* carbohydrate, or as much as two hours at 37 C in the case of the component of tubercle bacilli used by Middlebrook and Dubos (1948). In the case of the streptococcal substance described here the optimal conditions for adsorption lie well within this range.

A possible difference among these various adsorption-agglutination tests in terms of the structure of the erythrocyte wall involved is suggested also by the fact that human erythrocytes could be used in the case of antigens derived from *Hemophilus* and *Pasteurella*, but not in the case of the streptococcal substance involved in the current report.

The failure of tannic acid treatment of the red cells to increase the sensitivity of the test is not surprising in view of the presumable variety of bacterial products present in the streptococcal preparation used. In the work of Boyden (1951), solutions of single proteins were used, and any loss of specificity attendant on the increased sensitivity would not affect the results of serologic tests. However, in the present situation, the positive adsorption-agglutination test probably depends on an essentially fortuitous reaction between a component of the mixture of bacterial products, and a decrease in specificity concomitant with increased sensitivity might well interfere with the reaction.

The distinction between red cell adsorbed substance and CSA. Of the extracellular streptococcal antigens which had been described at the beginning of this study all were measured by their activity in some biologic or biochemical phenomenon, the relative concentrations of antibodies to these antigens being estimated in terms of their ability to neutralize those reactions. In contrast, all of the known somatic antigens of the hemolytic streptococcus are detected or estimated by direct combination of antigen and antibody, in aggregation phenomena or complement-fixation. At the time the work described here was undertaken there was also in progress a study of an antigen or complex of antigens found in streptococcal culture supernates and demonstrable by complement-fixation, designated as CSA. Because these were the only two currently known extracellular streptococcal antigens detected by aggregation or complement-fixation, and because they were both present in the same preparations, it was necessary to ascertain whether these were the same or different streptococcal products. The data presented would indicate that the substances detected and measured by the adsorption-agglutination test and by the complement-fixation test, respectively, are different streptococcal products.

Antired cell adsorbed substance titers of sera from patients with scarlet fever and rheumatic fever. The tests performed on pairs of sera from the same patients at the onset of scarlet fever and during convalescence from the disease indicated no rise in antired cell adsorbed substance titer

in the great majority of these patients. This result was rather unexpected because although streptococcal antibodies have been found to differ in terms of the frequency with which they show significant increases in titer in the course of streptococcal infection, this is the only streptococcal substance, to our knowledge, to which only rare increases in antibody titer have been found after streptococcal infection. It is of interest in this connection to note that Kirby, using available preparations of streptolysin O for adsorption of erythrocytes, also found no increase of adsorption-agglutination titers in the course of acute streptococcal infection.

The failure of the antired cell adsorbed substance titer in serum to increase during the course of scarlet fever, although the titers in the acute rheumatics varied from less than 6 to 128, would seem to indicate that red cell adsorbed substance is not antigenic in the short-term contact of acute infection but that cumulative effects of the repeated, usually subclinical, contacts of human beings with hemolytic streptococci may serve to provide effective antigenic stimulation of many subjects.

A final point may be mentioned in connection with the antired cell adsorbed substance titers found in acute rheumatic fever. Serologic studies with streptococcal antigens have in the past shown elevated serum titers both in patients with active rheumatic infection and those convalescing from acute streptococcal infection. Such observations, in conjunction with epidemiologic data, have been taken to support a hypothesis of some relationship of rheumatic fever to streptococcal infection. In the case of the antired cell adsorbed substance titers no elevation of range or mean titer was found in active rheumatic infection in comparison with a normal population; however, this finding is not inconsistent with a concept of a relation between rheumatic fever and the hemolytic streptococcus in view of the failure of patients with an acute streptococcal infection to show increases in antired cell adsorbed substance titers in the course of that infection.

SUMMARY

Incubation of sheep erythrocytes with concentrated filtrates of hemolytic streptococcal cultures renders them agglutinable by some human sera.

The material presumably adsorbed by the erythrocytes from such concentrates is distinct from a complement-fixing antigen or antigens found in such preparations of streptococcal culture filtrates.

In the erythrocyte adsorption-agglutination test described here, serum titers of patients have been found to increase only in rare instances between the acute and convalescent stage of scarlet fever and not to be elevated in active rheumatic fever.

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