SPECIFIC SERUM AGGLUTINATION OF ERYTHROCYTES SENSITIZED WITH EXTRACTS OF TUBERCLE BACILLI

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It is known that red blood corpuscles can adsorb various substances and thereby be rendered specifically agglutinable by serum antibody directed against the substance adsorbed (1–4). The present paper describes observations on the agglutination, by the sera of experimental animals and of tuberculous patients, of erythrocytes previously treated with aqueous extracts of tubercle bacilli or with products of their culture filtrate.

Materials and Methods

Preparation of Extracts of Tubercle Bacilli.—The H37Rv strain of human tubercle bacillus was cultivated on the surface of a liquid medium described in a previous communication (medium 7 in reference 5). After approximately 14 days of growth, the whole bacillary mass was filtered free of the culture filtrate and washed on the filter, first with distilled water, then with cold acetone. Following removal from the filter, the organisms were air-dried at room temperature in a glass container. All these operations were carried out under irradiation with ultraviolet light. The dried bacillary material was stored at room temperature or in a refrigerator.

Four grams of air-dried bacilli was suspended in 100 cc. of 88 per cent phenol (phenol liquefied, U. S. P. XIII, Mallinckrodt) and stirred by a magnetic stirrer at 35°C. for 20 hours; the suspension was centrifuged and the clear brownish supernatant fluid discarded. The sediment was resuspended in 100 cc. of fresh phenol solution and the same operation was repeated. The new sediment was twice resuspended in fresh phenol solution, stirred for only ½ hour each time, and centrifuged. It was then washed three times with 90 cc. aliquots of cold acetone in order to remove the phenol. Finally, the phenol-acetone—treated bacillary mass was dried and stored at room temperature.

The phenol-acetone—treated, dried bacillary material (0.5 gm.) was suspended with mortar and pestle in an isotonic aqueous solution (65 cc.) containing 0.55 per cent NaCl, 0.5 per cent Na_2HPO_4, and 20 per cent methanol. The methanol served to facilitate suspension of the bacilli as well as to prevent contamination. This suspension was stirred for 20 hours by a magnetic stirrer at 35°C. The extract was freed of most of the suspended bacillary bodies by centrifugation in an angle head centrifuge (Sorval type) at 10,000 R.P.M. for 30 minutes. The slightly opalescent extract was adjusted to a pH of 6.0, was dialyzed through ordinary sausage casing cellophane against tap water for 6 hours, and then against distilled water for 1 to 3 days. The solution in the cellophane bag was evaporated down to a volume of 20 cc. in front of a fan, removed from the bag, and centrifuged at high speed to yield an almost clear extract which was adjusted to pH 6.5 to 7.0 with dilute NaOH and made isotonic by the addition of 0.52 cc. of saturated aqueous solution of sodium chloride.

Preparation of Washed, Packed, Sheep Erythrocytes.—Sheep's blood was collected aseptically in 1.2 volumes of sterile modified Alsever's solution (6). The red cells in this mixture remain adequate for the hemagglutination test for 3 months when kept under aseptic conditions at

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4°C. The mixture of blood and Alsever's solution was centrifuged and the red cells were washed three times with 6 volumes of isotonic saline solution. After the last centrifugation at 2000 R.P.M. for 20 minutes, as much of the supernatant fluid as possible was removed and the packed cells were stored at 4°C.; these cells were used within 3 days or until hemolysis was clearly evident. Fifty cc. of blood-Alsever's mixture yielded about 7 cc. of packed, washed erythrocytes.

Sensitization of Sheep's Cells with Extract.—Approximately 0.5 cc. of packed, washed red cells was added to 10 cc. of the neutral, isotonic extract of tubercle bacilli; the cells were well suspended and placed in a water bath at 37°C. for 2 hours and frequently agitated; then the suspension was centrifuged and the clear supernatant fluid was discarded. The packed treated red cells were washed three times with 30 cc. of saline and finally suspended in 100 cc. of sterile saline (0.5 per cent "sensitized red cell suspension") to be stored at 4°C. and used within 3 days or until hemolysis was clearly evident.

Collection and Preparation of Serum for Absorption.—Blood was collected and allowed to clot, and the clot allowed to retract; serum was removed and, for preservation, when desired, merthiolate was added in a final concentration of 0.01 per cent. To 1 cc. of serum was added 1 cc. of saline and this mixture was heated at 56°C. for 30 minutes in order to inactivate complement.

Absorption of Serum with Untreated Red Cells.—The following operations were designed to remove from the sera, antibodies unrelated to tuberculous infection which would agglutinate untreated red cells by the hemagglutination technique to be described.

In 2 cc. of 1:2 dilution of heated serum 0.2 cc. of packed, washed, untreated sheep's cells was suspended, and the suspension was allowed to stand at room temperature for at least 20 minutes, during which it was frequently shaken. It was then centrifuged to sediment the cells and the supernatant fluid was removed and treated in the same way with another 0.2 cc. of packed untreated cells. The final supernatant fluid was used in the hemagglutination tests as representing a 1:2 dilution of absorbed serum.

A 0.5 per cent suspension of untreated red cells, to be used as control antigen in the testing of all sera, was prepared by suspending 0.1 cc. of untreated, washed, packed sheep cells in 20 cc. of isotonic sterile saline. This suspension was stored in the refrigerator and used no longer than 3 days after its preparation.

Performance of Hemagglutination Test.—Twofold serial dilutions of the absorbed serum to be tested were made in saline in tubes of 12 mm. internal diameter. To tubes containing 0.4 cc. of serum dilution there was added 0.4 cc. of 0.5 per cent suspension of treated erythrocytes. Two control tubes were found to be necessary: one containing 0.4 cc. of saline and 0.4 cc. of 0.5 per cent suspension of treated cells, and the other containing 0.4 cc. of 1:2 dilution of absorbed serum and 0.4 cc. of 0.5 per cent suspension of untreated erythrocytes.

Incubation at 37°C. for 2 hours, followed by a preliminary observation, a vigorous shaking, and further incubation at room temperature overnight, was found to allow satisfactory reading of the degree of agglutination. The results were read and recorded as in any red cell agglutination test.

Agglutination of the treated cells in saline has usually been present to an insignificant extent and this has served as a guide in the judging of clear cut agglutination in an actively agglutinating serum. Agglutination of untreated red cells by the absorbed serum indicates that the serum has been incompletely absorbed and renders the test invalid.

Technique of Inhibition of Specific Hemagglutination by Solutions Containing Sensitizing Material.—An active serum was selected which agglutinated treated red cells at high titre, and a serum dilution near the end-point of agglutination was carefully determined which consistently gave definite, though not strong, aggutination of treated red cells. Serum in this dilution was added in 0.4 cc. amounts to a series of tubes containing 0.4 cc. of serial dilu-

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tions of the substance to be tested for its ability to inhibit the specific agglutination. The tubes were incubated in a water bath at 37°C. for 45 minutes. To each tube was then added 0.4 cc. of a 0.5 per cent suspension of treated red cells. Incubation and the reading of results were carried out as in the case of the above described hemagglutination test. Appropriate controls containing untreated red cells with the undiluted solution to be tested, treated cells with saline, and treated cells with serum, respectively, were included in such tests.

EXPERIMENTAL RESULTS

Study of Sera.—Table I presents the results obtained in the examination of various active sera.

TABLE I

Agglutination of Sensitized Red Cells by the Sera of Rabbits Injected with BCG

Dilutions of sera in saline	Immune rabbit sera						Normal rabbit serum
	1 (742)	2 (744)	3 (745)	4 (734)	5 (735)	6 (736)	
1:10	++++	++++	++++	++++	++++	++++	−
1:20	++++	++++	++++	++++	++++	++++	−
1:40	++++	++++	++	++++	++++	++++	−
1:80	++++	++++	+	++++	++++	++++	−
1:160	+++	+++	−	++++	++++	++++	−
1:320	+	+	−	++++	++++	++++	−
1:640	−	−	−	+++	+++	++	−
1:1280	−	−	−	++	+	+	−
1:2560	−	−	−	−	−	−	−
1:10 with untreated red cells	−	−	−	−	−	−	−

Sera 1, 2, and 3 were drawn from rabbits which had received 8 weeks previously one single intravenous injection of 0.2 mg. of a living culture of a strain of BCG identified as BCG 317 (7). Sera 4, 5, and 6 were drawn from rabbits injected according to a different schedule: the animals were injected by the intravenous route with 0.2 mg. of a living culture of the same strain of BCG and were allowed to rest for 5 weeks; then three weekly injections of 0.02 mg. of the same culture in 1 cc. of saline were made into the left heart in order to distribute the bacilli into parts of the body other than the lung.

The data presented show that the titres of antibody against the treated red cells were consistently and significantly higher in the rabbits repeatedly injected with tubercle bacilli.

In Table II are recorded the results obtained with the sera of some patients with active pulmonary tuberculosis. No correlation has yet been attempted between the degree of activity of the disease and the titre of the serum in the hemagglutination test, but it is evident that all the patients tested had titres

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of 1:8 or higher; that is to say, they possessed antibodies capable of agglutinating the sensitized red cells. One additional serum from a patient with miliary tuberculosis, made available to us by Dr. Walsh McDermott of the New York Hospital, had a titre of 1:256.

Some evidence has been obtained for the specificity of the hemagglutination reaction by testing red cells treated with the extract of tubercle bacilli against the sera of experimental animals which had been immunized with other microorganisms:[1] Pneumococcus Type I, Pneumococcus Type III, Pneumococcus Type XIV, Friedländer bacilli types B and C, and Flexner dysentery bacilli types X and Y. There were weak cross-reactions with Friedländer antisera which gave doubtful agglutination at 1:2 dilutions of serum; these reactions can be considered insignificant. There were also weak reactions with high

TABLE II

Agglutination of Sensitized Red Cells by the Sera of Tuberculous Patients

Dilutions of sera in saline	Human sera						Saline control
	1	2	3	4	5	6	
1:2	+++	++++	+++	++++	++++	++++	−
1:4	++	++++	++	++++	++++	++++	−
1:8	+	++++	+	++++	+++	++++	−
1:16	−	++	±	+++	+++	++	−
1:32	−	±	−	++	++	+	−
1:64	−	−	−	+	±	−	−
1:2 with untreated red cells	−	−	−	−	−	−	−

titred Pneumococcus Type XIV antisera, both from the horse and from the rabbit; these were not marked, and appeared only in the dilution below 1:32.

Table III gives further evidence for the specificity of the reaction. Serum 1 was a pool of sera from 10 to 20 individuals all giving strongly positive Wassermann reactions for syphilis;[2] it is probable that many of these individuals were tuberculin-positive, but there was no evidence that any of them had active tuberculous disease. The pooled serum was also investigated for its ability, at a dilution of 1:4, to inhibit specific hemagglutination by a selected active serum, but there was no evidence of such inhibitory activity. Sera 2, 3, 4, and 5 were from patients with or convalescing from acute streptococcal infections.[3]

[1] Kindly supplied for our use by Dr. Walther Goebel and Dr. Frank Horsfall of the Rockefeller Institute.

[2] This pool was generously supplied by Dr. Widelock of the Serology Laboratories of the New York City Department of Health.

[3] Made available through the kindness of Dr. Sidney Rothbard of the Hospital of the Rockefeller Institute.

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Sera 6, 7, 8, 9, and 10 were from tuberculin-negative student nurses at the New York Hospital.[4] Serum 11 was from a tuberculin-positive individual who had been working for many months with living tubercle bacilli but had no evidence of active disease. As will be noted from the results presented in Table III, none of these sera gave titres higher than 1:4 in the hemagglutination test.

Study of Antigen.—Material from the tubercle bacillus which sensitizes red cells to agglutination in tuberculous antisera has been demonstrated to be present in at least one preparation of old tuberculin. Thus, one sample of Gilliland O. T. (Wyeth), which had been steamed for many hours during preparation, was observed to be as effective in sensitizing sheep erythrocytes as the extract described here. One ml. of this deglycerinated O. T.[5] was capable

TABLE III

Agglutination of Sensitized Red Cells by the Sera of Non-Tuberculous Individuals

Dilutions of sera in saline	Human sera											Saline control
	1	2	3	4	5	6	7	8	9	10	11	
1:2	±	−	−	−	−	++	−	−	−	++	+	−
1:4	−	−	−	−	−	±	−	−	−	+	−	−
1:8	−	−	−	−	−	−	−	−	−	±	−	−
1:16	−	−	−	−	−	−	−	−	−	−	−	−
1:32	−	−	−	−	−	−	−	−	−	−	−	−
1:2 with untreated red cells	−	−	−	−	−	−	−	−	−	−	−	−

of sensitizing completely 0.025 cc. of washed packed sheep erythrocytes to such an extent that they were agglutinated in the same way and to the same titre in an anti-BCG hyperimmune serum (No. 734) as were the cells of the same lot sensitized by the above described extract of tubercle bacilli. This fact shows that at least one substance responsible for sensitization is heat-stable and is present in the culture filtrate of cultures of mammalian tubercle bacilli.

Additional experiments were performed in an attempt to determine whether active material was present in the carbohydrate or in the protein fraction of the bacillary extracts and culture filtrates. Serial dilutions of these fractions were made in saline and were tested for their relative ability to inhibit specific hemagglutination of red cells sensitized by the extract of H37Rv. Table IV reveals the results of these experiments. Extract fraction 1 was the crude aqueous extract described in this paper. Extract fraction 2 was an aliquot of

[4] Made available through the kindness of Dr. F. Lansdown and Dr. C. Muschenheim of the New York Hospital.

[5] Available through the courtesy of Dr. Merrill Chase of the Rockefeller Institute.

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the same extract from which most of the protein had been removed by acidification with dilute HCl and repeated shaking with chloroform and isoamyl alcohol (8) until there was very little material at the chloroform-water interphase after centrifugation. It is evident that this procedure failed to decrease significantly the ability of the extract to inhibit specific hemagglutination even by a low dilution of antiserum.

TABLE IV

Inhibition of Specific Hemagglutination by Extracts and Fractions of Tubercle Bacilli

Dilutions of extracts in saline	Extracts and fractions							
	1*	2*	1‡	2‡	1§	2§	3§	4§
Undiluted	−	−	−	−	−	−	−	−
1:2	−	−	−	−	−	−	−	−
1:4	−	−	−	−	−	−	−	−
1:8	−	−	−	−	−	−	+	−
1:16	−	−	−	−	−	−	++	−
1:32	−	+	−	−	−	−	++	±
1:64	++	+++	−	−	−	−	++	+
1:128	+++	+++	−	−	−	−	++	++
1:256			−	−	−	−	++	++
1:512			−	−	−	−	++	++
1:1024			−	−	+	+	++	++
1:2048			−	−	++	++	++	++
1:4096			±	+				
1:8192			++	++				
Saline control	+++	+++	++	++	++	++	++	++

* Tested against 1:10 dilution of BCG rabbit antiserum 735, absorbed with untreated red cells.

‡ Tested against 1:320 dilution of BCG rabbit antiserum 735, absorbed with untreated red cells.

§ Tested against 1:320 dilution of BCG rabbit antiserum 734, absorbed with untreated red cells.

Unheated culture filtrate fractions 3 and 4, made available for our use by Dr. Janet McCarter Woolley, had been defined chemically as follows. Fraction 3 was known to contain only very little polysaccharide and approximately $20\gamma/0.4$ cc. of tuberculoprotein as determined by non-nucleic acid nitrogen content. Fraction 4, on the contrary, contained approximately $20\gamma/0.4$ cc. of tuberculopolysaccharide and no more than one part of tuberculoprotein to 19 parts of polysaccharide. It is evident that material active in the hemagglutination test was in the polysaccharide fraction and that amounts as small as $0.16\gamma/0.4$ cc. of solution could be detected by the technique of specific inhibition

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of hemagglutination. It is clear, also, that it is possible by these techniques to standardize a solution of any extract or product of the culture filtrates of tubercle bacilli, with respect to activity in sensitizing sheep erythrocytes for the specific hemagglutination test.

The Use of an Avirulent Culture of Tubercle Bacilli for the Preparation of Sensitized Sheep Erythrocytes.—The avirulent variant, H37Ra (8), of the virulent strain, H37Rv, has been investigated for its ability to yield material capable of sensitizing sheep erythrocytes and to inhibit the specific hemagglutination of erythrocytes treated with the extract of H37Rv. No significant differences of a qualitative nature have, thus far, been detected in these respects between the two variant strains by the use of the high titred experimental animal sera or of sera of tuberculous patients.

DISCUSSION

The observations described show that at least one heat-stable component present in a polysaccharide fraction of the tubercle bacillus, can be adsorbed onto sheep erythrocytes, rendering them specifically agglutinable by antibody directed against the adsorbed material. The antibody responsible for this hemagglutination test circulates in the blood of immunized animals and of human beings with active tuberculosis. The test exhibits a high degree of specificity and, in particular, does not give rise to any cross-reaction with Wassermann-positive sera as sometimes occurs in the case of the complement fixation reactions in tuberculosis (9).

The specific hemagglutination reaction can be inhibited by adding the soluble reactive antigen to the serum before introducing the sensitized red cells into the system. Under these circumstances the soluble antigen, if present in sufficient amount, combines with its corresponding antibody and prevents it from agglutinating the sensitized erythrocytes. Thus this inhibition test, when utilized together with the agglutination test proper, permits the detection and quantitation of very small amounts of the sensitizing antigen, as repeated observations have shown. It will be interesting to test whether this technique permits the detection of specific antigen circulating *in vivo*.

SUMMARY

A hemagglutination reaction has been described between sheep erythrocytes treated with a component of a polysaccharide fraction of mammalian tubercle bacilli and the sera of experimental animals or of tuberculous patients.

Evidence has been presented for the specificity of this reaction. A modification of the test, involving an inhibition reaction, has been developed for the detection and quantitation of minute amounts of the material responsible for the hemagglutination reaction.

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