A METHOD FOR COUPLING PROTEIN ANTIGENS TO ERYTHROCYTES

I. DESCRIPTION OF METHOD*

By LEON R. COLE,‡ M.D., AND VIRGINIA R. FARRELL

(*From the Departments of Medicine, Peter Bent Brigham Hospital, and Harvard Medical School, Boston*)

Allergy in tuberculosis is of a dual nature, in that anaphylactic type response occurs as well as the characteristic tuberculin reaction (1, 2). Enders (3) found that anaphylactic reactions appeared to be in response to a polysaccharide antigen. A large body of evidence, summarized by Drea and Andrejew (4), has indicated that delayed type tuberculin reaction is produced by a protein material.

A similar duality has been noted in the antibody response. A sensitive system for the measurement of antibody to bacterial polysaccharides was described by Keogh and his colleagues (5). They effected the adsorption, onto normal red blood cells, of polysaccharide from strains of *Hemophilus influenzae* and other bacteria. These sensitized cells were agglutinated by corresponding antisera. This technique has been applied to the study of tuberculosis by Middlebrook and Dubos (6), who employed an extract from tubercle bacilli as a sensitizing agent, and demonstrated that tuberculopolysaccharide could inhibit the hemagglutination more effectively than could tuberculoprotein. Middlebrook later obtained a highly purified polysaccharide which effects sensitization of erythrocytes (7).

Boyden (8), on the other hand, evolved an entirely different type of hemagglutination technique, whereby diluted purified protein derivative (PPD) is adsorbed onto red blood cells pretreated with tannic acid. Grabar and his associates (9) found that tuberculoprotein inhibited this antibody more powerfully than did tuberculopolysaccharide and demonstrated, with cross-adsorption studies, that it is unrelated to the Middlebrook-Dubos antibody. Meynell (10) was able to remove the latter antibody from serum by adsorption with a suspension of whole *Mycobacterium tuberculosis*, whereas the Boyden titer remained unchanged.

Correlations between *in vivo* and *in vitro* methods were made by Cole and Favour (11). They fractionated large volumes of immune guinea pig plasma and injected the separate fractions into normal animals. The recipients were skin-tested with a variety of derivatives of tubercle bacillus. The gamma globulin effected passive transfer of

anaphylaxis and urticarial type skin reactivity to tuberculopolysaccharide, and contained the entire Middlebrook-Dubos titer of the plasma. Antibody to tuberculoprotein was found exclusively in the alpha globulin, which contained the entire Boyden titer of plasma and passively transferred a delayed type of skin reactivity to tuberculin PPD.

The present study has attempted to provide a convenient method for the measurement of antibody to tuberculoprotein in human serum. It is, of course, appreciated that completely "pure" preparations of protein and polysaccharide are not available, and that the chemical constituents of the tubercle bacillus are of a complex nature (12).

Tuberculin PPD has been coupled, by tetrazotized benzidine, to formalinized erythrocytes. These cells, when added to immune serum, are agglutinated by antibody to PPD. The sensitized, formalinized cells are stable. Prepared in large volumes, they may be dispersed into small ampoules and stored in a frozen state until used.

EXPERIMENTAL

Phosphate Buffer.—0.15 M Na_2HPO_4 and KH_2PO_4 were mixed in proportions to give pH 7.3 (1075 ml. Na_2HPO_4 and 295 ml. KH_2PO_4). To 2 liters of buffer was added 0.32 ml. of 10 per cent ethylenediaminetetraacetate (EDTA).[1]

Buffered Saline.—Equal parts of 0.85 per cent NaCl and phosphate buffer.

Sodium Ethylenediaminetetraacetate.—Disodium versenate[2], analytical reagent grade was used. It was brought to pH 7.3 with 2.5 N NaOH and kept as a 10 per cent solution in a polyethylene bottle.

Tuberculin PPD[3].—Tuberculin PPD was used in concentration of 1 mg. per ml. Ampoules of 40 mg. active principle were diluted with 40 ml. saline, divided into smaller bottles, and kept at −12°C. Parke, Davis & Company PPD is prepared by the method of Seibert (13).

Polysaccharide I[4].—Isolation of this polysaccharide is discussed by Seibert and Watson (14) and Tennent and Watson (15). It contains 0.85 per cent N. A concentration of 1 mg. per ml. was used.

Formalinized Erythrocytes.—*Outdated* Group A bank blood was treated with formalin by the method of Flick (16). 100 ml. of 37 per cent formalin was added to 100 ml. of a 50 per cent suspension in buffered saline of washed cells, and kept at 2°C. for 3 days. The formalin was then removed by washing 10 to 12 times over a 5 day period. The first washings were in saline and the last three in buffered saline. The cells were mixed for 1 minute in a Waring blendor after the first washing, to eliminate their gel-like consistency. They were finally prepared in a 50 per cent suspension, stored at 0–2°C. and used for 2 months.

Normal Rabbit Serum.—This was used as diluent of both sensitized cells and test sera. For each preparation of cells, serum from the same rabbit was used as diluent for test sera. Complement was inactivated by adding 0.4 ml. of 10 per cent EDTA per 10 ml. serum. For each 15 ml. of serum, 6 adsorptions with a total of 3 ml. packed unsensitized, formalinized cells were

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[1] EDTA, ethylenediaminetetraacetate.

[2] Kindly supplied by Bersworth Chemical Co., Framingham, Massachusetts.

[3] Kindly donated by Parke, Davis & Company, Detroit—lot 026269—B.

[4] Kindly given to us by Dr. Dennis W. Watson, Department of Bacteriology, University of Minnesota, Minneapolis.

performed. Slight hemolysis occurred with each adsorption. Normal rabbit serum was discarded after 7 days.

Normal Guinea Pig Serum.—This was prepared from the pooled sera of at least five animals. It was treated as was the normal rabbit serum.

Immune Human Sera.—These were obtained from patients with active tuberculosis. Bloods were centrifuged at 4°C. within 2 hours after they were drawn. The serum was kept at 0–2°C. Complement was inactivated by adding 0.04 ml. of 10 per cent EDTA per 0.4 ml. serum.

Normal Human Sera.—These sera were obtained from non-tuberculous patients and were treated in the same way as the immune sera.

Preparation of Tetrazotized Benzidine.—23 mg. benzidine base (reagent)[5] was dissolved in 7.5 ml. 1 N HCl at room temperature, and cooled to 0–1°C. in an ice water bath. To this was added, rather slowly, 1.25 ml. of precooled 0.2 N $NaNO_2$. The solution was used 10 minutes later and had developed a yellow color in that time.

Preparation of Erythrocytes.—Two techniques, differing in certain particulars, were assayed. They are described below as method A and method B. Method A was devised later than method B, and had several improvements.

Method A: 30 ml. of stock formalinized erythrocyte suspension was mixed in a Waring blendor (head No. 17246B) for a total of 60 minutes. The cells were mixed for 30 minutes on the day before sensitization and 30 minutes on the same day, or for the entire 60 minutes on the day of sensitization. A loose paper jacket was placed around the blendor head, and a few small pieces of dry ice were inserted. The cells were thereby maintained in a cool state during the entire homogenization.

About 15 ml. of the homogenized cells was placed in a graduated conical 15 ml. test tube. It was centrifuged at 1600 R.P.M. for 15 minutes at 0°C. The slightly cloudy supernatant was discarded. Exactly 4 ml. of packed cells was desired. Corrections of volume were made by adding or removing cell suspension and centrifuging another 3 minutes.

Sensitization of Erythrocytes.—An ice water bath was prepared, and continuous circulation maintained with a mechanical stirrer. All reagents used in this procedure were thereby kept at 0–2°C. Centrifugings were performed in a refrigerated centrifuge at 0°C.

The 4 ml. of packed cells was brought to 40 ml. with buffered saline pH 9 (0.15 M Na_2HPO_4 and 0.85 per cent NaCl in equal parts). To this was added 4 ml. of the tetrazotized benzidine with stirring. The suspension was agitated frequently. Initial pH of this suspension was 6.7, and it was quickly raised to approximately pH 9 with NaOH (about 1.48 ml. of 2.5 N NaOH). 30 minutes after the pH had been adjusted, the cell suspension was divided equally in 4 conical 50 ml. centrifuge tubes. Each tube was brought up to 40 ml. with buffered saline pH 7.3. The tubes were centrifuged at 1800 R.P.M. for 10 minutes and the supernatants discarded. To each tube were then added 1.5 ml. tuberculin PPD and 2 ml. buffered saline pH 7.3. The suspensions were mixed and pooled. The pH at this point was usually 7.5 and was adjusted to 7.3 with a couple drops of 1 N HCl (a minimal aliquot was diluted with 0.02 N NaCl for all pH determinations). The suspension was then redivided into two tubes and the pH was checked every hour. After 2 hours, the tubes were centrifuged at 1800 R.P.M. for 4 minutes and the supernatants discarded. Four washings were done with 40 ml. of 0.5 per cent NRS in buffered saline. Each centrifuging was at 1800 R.P.M. for 6 minutes.

After the fourth washing, both tubes were again diluted to 40 ml. with 0.5 per cent NRS in buffered saline. The suspensions were mixed thoroughly by drawing up and down in a capillary pipette. They were then placed at 2°C. for 1 hour and at room temperature for another hour. Sedimented cells formed a level of 0.2 to 0.4 ml. in the 2 hour period. The upper 39 ml. of suspension was removed from each tube and pooled. The lower 1 ml. was discarded.

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[5] Hartman-Leddon Company, Philadelphia.

The final volume of the pooled, sensitized cells was adjusted to 180 ml. with 0.5 per cent NRS in buffered saline. The cells were dispersed into 2 ml. ampoules, frozen in dry ice–alcohol, and stored at −40°C.

Method B: In this method, the formalinized erythrocytes were homogenized for only 20 minutes, on the day of sensitization. The sensitization technique was identical with method A. The step in which clumped sensitized cells were allowed to sediment out of suspension was
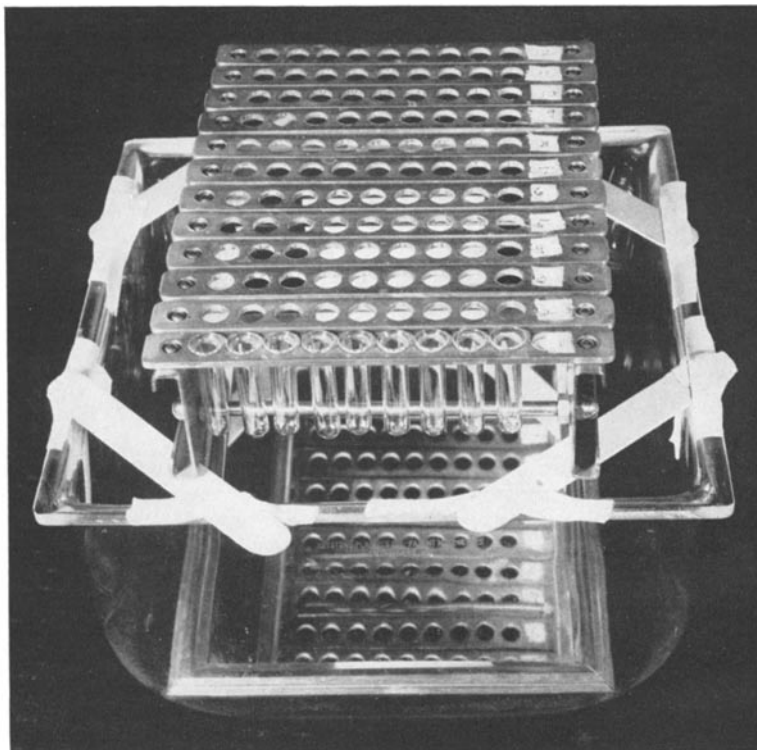

Fig. 1. A test tube rack containing 12 removable strips. The first strip contains 9 tubes which are suspended from their rims. The rack is placed on a reading box with a mirror which permits viewing of the bottoms of the tubes.

different. The tubes were allowed to stand until the sediment was 1 ml., though the time required was consistently 2 hours. This 1 ml. was discarded. The final volume of sensitized cells was 60 ml., and the preparation was kept at 2°C. With each use, the supernatant was changed and the suspension was rehomogenized 10 to 20 minutes. NGPS[6] was employed instead of NRS[7] as diluent.

Test Tubes and Racks.—Special small tubes were used for the hemagglutination test, and they were held in a metal rack[8] which is shown in Fig. 1. The tubes were 10 × 50 mm. with

[6] NGPS, normal guinea pig serum.

[7] NRS, normal rabbit serum.

[8] Made to order by B. Feneno Company, Boston.

perfectly hemispherical bottoms. Their inner diameter was about 8.5 mm. They were lipped so as to hang from the holes of the metal strips.

The metal racks were 10 x 7⅞₆ inches and each rack held 12 strips, 7½ x ¾ inches. The strips contained 10 holes for tubes, ⅝₆ inch in diameter. A hole at each end, of diameter ¼ inch, was fitted into its corresponding peg along the sides of the rack. The racks were placed on a clear plastic box with a mirror which reflected the patterns in the bottoms of the tubes.

The tubes were cleaned by wrapping a rubber band about bunches of 18 tubes, emptying their contents, and submerging in calgolac detergent overnight. They were scrubbed with cotton applicator sticks, and then rinsed several times in tap water and distilled water. Finally the rubber bands were removed and the tubes were dried.

Performance of Test.—For each serum to be tested, 1 conical graduated tube and seven small culture tubes were placed in a row. To the conical tube was added 0.4 ml. of serum, 0.04 ml. of 10 per cent EDTA, and 0.15 ml. of normal rabbit serum (NRS). The volume was then brought to 2 ml. with 1.5 per cent NRS in buffered saline. The other tubes received 1 ml. of 1.5 per cent NRS in buffered saline. Twofold serial dilutions were made by transferring 1 ml. down each set of tubes. From every serum dilution, 0.5 ml. was pipetted into each of two corresponding small tubes in the special rack. Duplicate aliquots were thereby obtained.

A ninth tube, containing 0.5 ml. of 1.5 per cent NRS in buffered saline was included as a control.

At the end of this study the twofold dilutions were made directly in the special small tubes in the racks. This was done with a 1 ml. tuberculin syringe firmly affixed with a plastic tube to a No. 18 needle. The needle had been shortened to 60 mm., and most of its head sawed away to eliminate dead space. The syringe was washed between sera by filling it several times with 0.85 per cent NaCl, and discarding the contents.

The racks containing the serum dilutions were placed in the refrigerator for 30 to 60 minutes. During this time the refrigerator was adjusted to 0–1°C. and allowed to stabilize.

Ampoules of frozen, sensitized cells were thawed in cool water. The suspensions were transferred to test tubes, kept in ice water, and used within 30 minutes. The precooled racks of tubes were removed from the refrigerator. Using a calibrated pipette, 1 drop (0.034 to 0.040 ml.) of cell suspension was added to each tube. The metal strips, each holding nine tubes, were shaken individually to mix the cells thoroughly. The racks were returned immediately to the refrigerator and left undisturbed overnight. Hemagglutination patterns were read in a manner fairly similar to the Boyden technique (8). Readings were completed within 20 minutes of removal from the refrigerator.

RESULTS

Preparation of Antigen

Homogenization of Formalinized Erythrocytes.—Formalinized erythrocytes existed in clumps that could be dispersed by mixing in a Waring blendor. No hemolysis resulted from the mixing, and few cell fragments occurred. After 20 minutes of homogenizing, as in method B, many clumps of 2 to 7 cells were present. Homogenization for 60 minutes, as in method A, reduced the suspension almost entirely to individual cells. There was little tendency for reclumping. The Waring blendor was taken apart and cleaned frequently.

Treatment of Formalinized Erythrocytes with Tetrazotized Benzidine.—The tetrazotized benzidine was prepared in a manner similar to that of Coombs and his associates (19). This agent couples proteins mainly through linkages with their histidine and tyrosine groups. A large amount of tetrazotized ben-

zidine appeared to coat the formalinized cells, changing their color from chocolate brown to light red.

To aliquots of tetrazotized benzidine-treated, and untreated, formalinized cells was added a small amount of Evans blue. After 2 hours at 0°C., the untreated cells were blue whereas the treated cells were intensely green. Twelve washings with 50 per cent normal serum in saline sufficed to remove the Evans blue from the untreated cells, whereas the treated aliquot had lost only a little of its green color.

It appeared probable that the Evans blue had been coupled onto the treated cells, and the green color was a combination of the yellow tetrazotized benzidine and the blue dye. The small amount of dye that was washed from the treated cells was blue.

Since coupling proceeds most rapidly in alkaline solution, the cells were treated at pH 9. No clumping occurred under the conditions of the present technique.

Sensitization of Treated Formalinized Erythrocytes.—The tyrosine content of tuberculin PPD, by the method of Folin and Ciocalteau (17), was found to be 6.7 per cent. It thus appeared that this antigen could be coupled onto treated cells. The 6 mg. of PPD used for each 4 ml. of packed cells represented an attempt to provide an excess. No effort was made to determine the minimum quantity of PPD necessary. Experiments were performed to demonstrate the presence of PPD on the cells at the end of the coupling procedure.

Suspensions of sensitized and unsensitized formalinized red blood cells were diluted until faintly cloudy in appearance. These were injected intradermally, in doses of 0.1 ml., into a subject known to react strongly to PPD. There was some impaction of red cells in the needles but enough of the cells were deposited in the skin to produce tiny red marks. Clear supernatants, obtained by centrifuging the suspensions, were also assayed by skin tests. At 24 hours the sensitized cells produced a typical skin reaction which was, however, smaller than the response to first strength PPD. The supernatant fluid and the unsensitized formalinized cells were negative. The latter did develop some inflammation in 48 hours. This was thought to be non-specific.

Immune sera were tested against (a) PPD-coupled, formalinized cells, and (b) formalinized cells which had been exposed to PPD for 2 hours under the same conditions of concentration, pH and temperature as the former, and then washed 4 times to remove excess PPD. Table I demonstrates the comparative results.

The tuberculin PPD was coupled at pH 7.3. This pH appeared somewhat critical. Four different sensitization experiments were performed at pH 9 and all four of the resulting cell preparations failed to contain demonstrable PPD. The procedure at pH 7.3 was done more than 24 times with uniform results. The use of 0.5 per cent NRS or 0.5 per cent NGPS diluent was a standard procedure which was not varied.

Sedimentation of Sensitized, Formalinized Erythrocytes.—The sensitized cell preparations were allowed to stand for 2 hours to allow small clumps to settle

at the bottom. Considerably more sediment occurred in method B than in method A and microscopic examination revealed larger clumps. This was expected in view of the respective homogenization times. The final suspension consisted almost entirely of individual cells by method A, whereas a significant

TABLE I

Comparison of Titers Obtained with Cells Coupled to PPD and Exposed to PPD

Serum	Titer	
	PPD coupled	PPD exposed
1	40	0
2	20	0
3	160	5
4	80	0
5	160	5
6	40	0
7	0	0
8	80	0
9	40	0
10	20	0

TABLE II

Correlation of Antibody Titer with Concentration of Sensitized Cells

Patient	Titer	
	Volume of drops, *ml.*	
	0.055	0.038
1	5	640
2	40	160
3	5	80
4	0	40
5	160	320
6	10	40
7	5	80

number of small clumps (2 to 4 cells) were present in the method B preparations.

Spontaneous clumping of sensitized cells occurred overnight in the refrigerator. For this reason, the suspensions as employed in method B required re-homogenizing before each use.

Concentration of Sensitized, Formalinized Cells.—The quantity of sensitized cells added to each tube of test serum was critical. This quantity was a function of three factors: (a) concentration of the sensitized cell suspension, (b) volume

of the suspension dispensed to each tube, and, (c) amount of clumping in the cell suspension. Thus, since the cells obtained by method A were more thoroughly dispersed than those of method B, and other factors were constant, the optimal volume of the former suspension proved to be 180 ml., against 60 ml. for the latter.

Decreased sensitivity of hemagglutination occurred when cells were added in too great concentration. Table II demonstrates the differences in titers of sera from tuberculous patients when the drop of sensitized cells added to each tube was 0.055 ml., compared with 0.038 ml. The latter volume appeared superior. Both positive and negative patterns were less distinct when the larger volume was used.

Irregular hemagglutination patterns occurred when the concentration of cells was too low, giving false positive results.

Preparation of Sera

Normal Rabbit Serum (NRS) and Normal Guinea Pig Serum (NGPS).—The 1.5 per cent NRS or NGPS in buffered saline had a definite stabilizing effect on the hemagglutination patterns, as compared with plain buffered saline. No advantage was found to 2 per cent solution. Other concentrations of diluent serum were not studied.

Addition of 0.15 ml. of NRS (NGPS in method B) to each 0.4 ml. of test serum resulted in more clearly defined positive and negative hemagglutination patterns.

A slight amount of lysis occurred during each of the 6 adsorptions of NRS. Hemolysis in the NGPS was appreciably greater. Several investigations of this phenomenon were made.

Using a method described by Cole and Favour (11) it was determined that all Ca and Mg in the serum had been chelated by versene. Multiple adsorptions with either sheep or Group A erythrocytes failed to prevent the subsequent lysis of formalinized cells. Adsorption of sera with formalinized red cells accomplished removal of their agglutinins.

Ethylenediaminetetraacetate.—EDTA was added to all sera for two purposes: (a) inactivation of complement, and (b) chelation of divalent metals to provide uniformity of cation composition.

Performance of Test

In previous sections is discussed the preparation of antigen and sera. The performance of the test was attended by one critical factor, the temperature. It was necessary to maintain the tubes constantly at 0–2°C. after the cell suspension had been added. At higher temperature, negative patterns often occurred in sera that otherwise had significant titers. This difference appeared related to a more rapid sedimentation of the sensitized cells at temperatures

above 2°C. It also appeared likely that changes in temperature produced convection currents that caused distortions of patterns.

The hemagglutination patterns were customarily read after 16 to 18 hours. Small non-specific irregularities began to appear after 24 hours. When once removed from the refrigerator, the tubes were read promptly. This was necessary because of progressive changes in the hemagglutination patterns which began 15 to 20 minutes after exposure to room temperature. These changes were due to a sliding inward of the ring patterns. They caused the disappearance of weekly positive patterns and distorted slightly the negative rings.

Reading of Hemagglutination Patterns

The characteristic hemagglutination patterns are shown in Figs. 2 a, 2 b, and 3. Reflections of light appear in the photographs and must not be confused with the erythrocytes. As previously described, nine tubes in a row were prepared for each serum. These contained twofold serum dilutions as follows: 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and, in the ninth tube, diluent as a control.

Negative Hemagglutination Patterns.—The hemagglutination patterns were similar to those found in the Boyden technique, with certain differences. Three types of negative patterns were observed. These were buttons, symmetrical rings, and forms midway between the two. The buttons tended to occur in the first five tubes. Symmetrical rings were present less frequently than buttons in the first five serial dilutions and were constantly seen in the higher dilutions. Buttons occurred less frequently in method B.

Button patterns were characterized by concentration of the erythrocytes centrally, with a relatively wide peripheral halo of more thinly packed cells. Their texture gave an impression of softness. The central portions of the buttons sometimes were irregular in appearance, when the red cells failed to converge into a uniform mass. These central irregularities were different from the peripheral irregularities of positive patterns.

The patterns midway between buttons and rings appeared unique to the present system. They were rings in which foldings inward of portions of the circumference produced irregularities. They only occurred in the tube immediately following a button, and appeared to represent transitions between the latter and rings. Occasionally they occurred in two or three consecutive tubes. In this case the irregularities were progressively less marked until a symmetrical ring was achieved. These transitional forms were sometimes indistinguishable from positive patterns if not appreciated in their relationship to the negative buttons. They were not dependent upon serum concentration since they might occur, for example, in the 1:10 dilution if the 1:5 contained a button, or in the 1:80 dilution if the first four tubes were all buttons. Titers were terminated by the first negative pattern to appear in a row of tubes. Since buttons

always preceded transitional forms, the latter did not create problems in the reading.

The symmetrical ring forms sometimes occurred throughout all the serum dilutions. Each serum, when tested repeatedly, appeared quite constant in its proportion of rings to buttons.
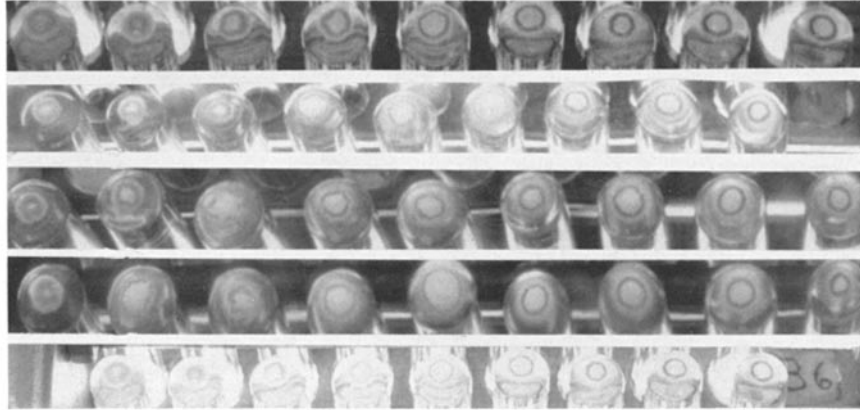

FIG. 2 a. These five rows demonstrate negative tests. Transitional forms of varying irregularity may be seen between the buttons and the rings.
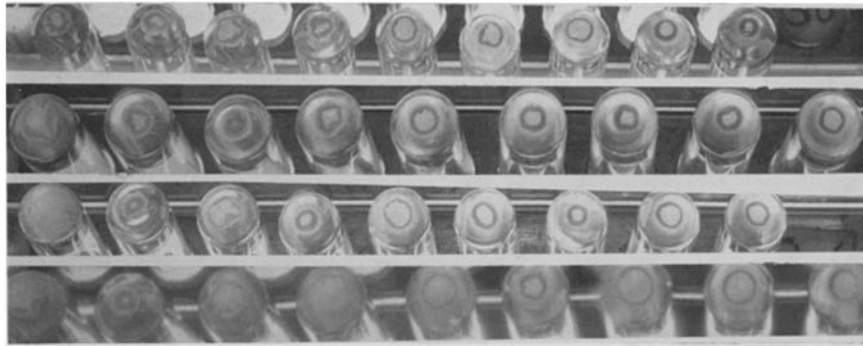

FIG. 2 b. These four rows demonstrate titers of 1:5. Tubes with transitional patterns may be seen between the tubes with buttons and those with rings. See text for discussion.

Fig. 2 a demonstrates five negative sera. Rows 1 and 2 each have buttons in the 1:5 and 1:10 dilutions, followed by two tubes with slightly irregular transitional forms. Row 3 likewise has buttons through the 1:10 dilution and in the 1:20 dilution is an unusually irregular transitional pattern, followed by another which is less pronounced. Row 4 is similar to row 3 except that a button is present only in the first tube. Row 5 has buttons through the 1:20 dilution and is

unusual in showing moderately irregular transitional patterns through the 1:320 dilution.

Fig. 2 *b* illustrates 4 sera with titers of 1:5. The first tube in each row contains a positive pattern and the next tube has a button The necessity of reading titers at the first negative button is herein demonstrated. Rows 1 and 3, with

Fig. 3. These nine rows demonstrate positive patterns in varying titers. See text for description.

buttons in both tubes 2 and 4, have transitional forms in tubes 3 and 5. Row 2 has buttons in the 1:10 and 1:20 tubes, followed by a transitional form of moderate irregularity. Row 4 shows two unusually distorted transitional patterns in the third and fourth tubes.

Row 1 in Fig. 2 *b* demonstrates, in the 1:5 dilution, a type of positive pattern which was somewhat similar to the negative buttons. It differed from the latter in that the erythrocytes were concentrated in the periphery, forming an irregular, often serrated ring. There was a granular appearance in contrast to the softness of negative buttons. Tubes 2 and 4 in this row show the irregularities that sometimes occurred in the central portions of negative buttons.

Positive Hemagglutination Patterns.—These took the form of irregular patterns in the gross, rings with serrations, thin blankets, or combinations of the three. Sometimes the positive patterns displayed a superficial resemblance to negative buttons, but, as mentioned above, the erythrocytes were concentrated in the periphery rather than centrally. They had varying degrees of irregularity, and a granular consistency. Such patterns are demonstrated in Fig. 3 as follows: the third tube in rows 4, 5, and 7; the second tube in rows 2 and 8, and the first tube in row 9.

Fig. 3 shows all the forms of positive patterns. No effort was made to grade the degree of positivity. In the dilutions of from 1:160–1:640, positive patterns generally took the form of irregular rings, or serrations that did not involve the entire circumference or else were thin blankets which coated the bottoms of the tubes. The higher concentrations of serum had in the gross more irregular

TABLE III

Heat Lability of Antibody

Patient	Titer	
	56°C. for 30 min.	EDTA
1	0	80
2	0	80
3	0	80

clumps of cells. Rows 1 and 2 have titers of 1:640+ and the 1:160, 1:320, and 1:640, rings were positive by virtue of symmetrical serrations plus small irregularities in shape. These serrations are somewhat indistinct in the photographs. Row 3 has a titer of 1:320. Rows 4, 5, 6, and 7 are 1:160. Row 8 is 1:40, as the 1:80 tube merely has a folding inward of part of the ring, which is not angular and has a soft texture. Row 9 has a 1:20 titer.

Positive patterns always dispersed readily on gentle shakiug, with no clumping of the cells.

Effect of Formalin on Blood Group Substance

Treatment of erythrocytes with formalin appeared to inactivate blood group substance. The incidence and extent of agglutination of unsensitized cells were the same as are shown in Table I in the PPD-exposed column.

Lability of Immune Serum

The lability of immune sera under conditions of heating or freezing was not a part of the present study. Under the conditions of the present technique, it was demonstrated in several instances that titers were reproducible for at least 2 weeks. Customarily, however, sera were examined within 2 days. In three

instances, comparison was made between inactivation of complement by EDTA and at 56°C. for 30 minutes. Results are shown in Table III and might suggest that heat depressed the antibody response.

Studies of Specific Inhibition

Experiments were performed to compare tuberculin PPD and polysaccharide I as specific inhibitors of hemagglutination by the present technique. These two materials were added to aliquots of immune serum. The latter were then prepared in serial twofold dilutions and agglutination of the PPD-coupled, formalinized red cells was measured. It was found that tuberculin PPD had a far greater capacity for inhibition of the hemagglutination than did polysaccharide I.

DISCUSSION

Pressman and his associates (18) employed tetrazotized benzidine to couple normal erythrocytes with protein antigen. They observed that the red blood cells could then be agglutinated by antibody to the corresponding antigen. However, the erythrocytes underwent spontaneous lysis 2 hours after they were exposed to the coupling procedure. Coombs and his associates (19) coupled egg albumin, and chicken serum globulin, to incomplete Rh antibody via tetrazotized benzidine. This coupled material could be frozen and stored. The incomplete Rh antibody retained its affinity for corresponding Rh-positive erythrocytes, sensitizing them with the egg albumin or chicken globulin. Specific antibody to these antigens produced hemagglutination.

The present technique has utilized the durability of formalinized erythrocytes to provide a stable antigen. The technique for preparation of antigen proved to be fairly simple, and a large quantity could be made at one time. Sensitized, formalinized cells were stored at −40°C. for 6 weeks with no gross sedimentation within the ampoules. Microscopic examination revealed no clumping after they were thawed. The maximum duration for storage was not determined. Glycerol might prove useful in preserving the cells.

Facility in the preparation of serial serum dilutions was obtained from compact small racks with miniature test tubes. The tubes were read with a mirror which permitted simultaneous visualization of 12 rows of tests. This system appeared to offer an improved perspective of the hemagglutination patterns.

Method A proved superior technically to method B. It appeared to achieve maximal dispersal of clumps, and was therefore a more uniform preparation. Method B required rehomogenization of cells with each use, because of the spontaneous clumping of sensitized cells that occurred at 2°C. About 4 ml. of suspension was lost through foaming with each mixing. The loss of cells was disproportionately greater than diluent, and resulted in a progressively more dilute cell suspension.

The lysis of formalinized erythrocytes in rabbit and guinea pig serum, though not striking, was not readily explained. If due to an antibody, it could not be removed by six adsorptions even though agglutinins were thereby removed. If complement was a factor, it could not be inactivated with EDTA. Adsorptions with sheep erythrocytes indicated that Forssmann antigen was not involved.

The fact that a highly dilute suspension of PPD-sensitized cells could produce a significant intradermal reaction might indicate that their surfaces contained a high concentration of PPD. It would be of interest to assay several protein fractions of the tubercle bacillus for their ability to sensitize the tetrazotized benzidine–treated cells, and correlate with their skin test activity.

Isliker (20) noted that red blood cell stroma treated with 0.5 per cent formalin loses avidity for isoagglutinins. It would appear that treatment with 18 per cent formalin causes quite a complete inactivation of group substance.

The inhibition studies demonstrated that tuberculin PPD blocks agglutination more effectively than does polysaccharide I. It thus appears that the antibody measured by the present technique is directed toward tuberculoprotein. It would be pertinent to assay immune plasma fractions for their content of this antibody, as was done by Cole and Favour (11) in demonstrating the differences between Middlebrook-Dubos and Boyden antibodies.

The present technique could not be readily applied to the detection of prozones. By definition, titers were read at the first negative hemagglutination pattern to occur in a row of serial dilutions, in order to avoid confusion from the negative transitional forms. The incidence of prozone phenomena in a hemagglutination system of this sort is not known but probably is not common. It would none-the-less be useful to eliminate the transitional type of pattern. This may be possible by changing the animal species used for diluent serum, or by alterations in the electrolyte content or specific gravity of the system.

SUMMARY

A technique has been described whereby tuberculin PPD is coupled, via tetrazotized benzidine, onto the surfaces of formalinized red blood cells. A stable antigen preparation is thereby obtained, which may be stored at −40°C. The purpose of the present method is to facilitate the study of antibody to tuberculoprotein.

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