

THE SPECIFICITY OF THE PASSIVE HEMAGGLUTINATION METHODS USED IN SEROLOGY OF TUBERCULOSIS

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As is well known, two different methods of passive hemagglutination are used in the study of tuberculosis: one is the Middlebrook-Dubos test (referred to as M.-D. test hereafter) using normal red cells (1948) (15) and the other the Boyden test (referred to as B. test hereafter) using red cells tanned with tannic acid (1951) (1).

The former is generally considered to be concerned in the antigen-antibody system of polysaccharides and the latter in that of proteins (2, 4, 14, 17-19, 21, 24, 26, 27, 34).

However, opinions still differ as to the specificity of the two tests. In a Ciba Foundation symposium on experimental tuberculosis (1955), Iland questioned the validity of the so called cross-inhibition test by which the specificity of serological reactions of this kind is generally tested (10). According to him, Pound (1955) obtained only variable results in testing the serological specificity of his fractions by inhibition tests (20). Shindo and Wakakura (1952) reported that their crystalline ovalbumin could sensitize normal sheep red cells to hemagglutination in the presence of homologous serum without pretreatment of the red cells with tannic acid (23). Hiyama (1958), using several tuberculin polysaccharide and purified protein fractions, did not find any important difference in absorbing ability between normal sheep red cells and sheep red cells tanned with tannic acid. Neither could he distinguish the B. test from the M.-D. test by cross-inhibiting his fractions; he therefore denied the specificity of the B. test for proteins (9).

It has been observed in this laboratory that the majority of polysaccharide and protein fractions isolated from tuberculins and tubercle bacilli and chemically purified are capable of sensitizing the two kinds of red cells, normal and tanned. In this case, one can not say whether the reaction really involves polysaccharide antibodies or protein antibodies. This fact, together with the discrepancies considered above, led the present authors to study the specificity of the two different passive hemagglutination tests, Middlebrook-Dubos' and Boyden's. For this purpose more than 20 different polysaccharide and protein fractions were isolated from tuberculins and tubercle bacilli in this laboratory. They were all subjected to the two tests and the data were compared with their chemical properties. It was found from these experiments that the ability of the fractions to sensitize normal and tanned red cells differed from one fraction to another according to their polysaccharide and nitrogen contents. The

polysaccharide fractions of which the nitrogen content was less than 1 per cent sensitized only normal red cells, while the protein fractions containing less than 1 per cent of polysaccharide sensitized only tanned red cells. Polysaccharide fractions containing more than 1 per cent of nitrogen and protein fractions containing more than 1 per cent of polysaccharide were found to be of dual character, being capable of sensitizing both normal and tanned red cells. Depending on the method of isolation, some fractions were found to be lacking in hemosensitizing ability, regardless of their nitrogen or polysaccharide contents. These were fractions extracted with NaOH or purified with active carbon powder and protein fractions precipitated from tuberculin at pH 2.2.

Recently, Takahashi (1958) and his collaborators have found that the phosphatide of the tubercle bacillus so far considered to be lacking in hemosensitizing ability (see review by Gernez-Rieux, 5) is capable of sensitizing normal sheep red cells to give hemagglutination reactions in the presence of tuberculous serum, and that the antigen-antibody system participating in the phosphatide hemagglutination is another one completely distinct from that participating in the polysaccharide hemagglutination (28-32).

These findings suggest that there exist in tuberculous serum three different kinds of antibodies: antiprotein, antipolysaccharide, and antiphosphatide. Efforts were therefore made further to separate the respective antibodies by the use of the antibody-absorption technique. By using absorbed antisera, the specificity of the three different passive hemagglutination tests was established. An abstract of the present study has already been reported (33).

Materials and Methods

1. *Antigens.*—Somatic protein fractions were extracted from defatted tubercle bacilli with 10 per cent urea solution. The extraction was done at room temperature for 4 to 5 consecutive days under constant agitation by means of a magnetic stirrer. After the bacilli were centrifuged off, the supernatant urea solutions were dialysed against running tap water for 2 consecutive days. Proteins were precipitated from the dialysed solutions by addition of a sufficient amount of 10 per cent trichloroacetic acid. The precipitates were collected by centrifugation, vacuum-dried, and purified by repeating solution in 0.1 M NaOH solution and precipitation with diluted hydrochloric acid. They were finally collected by centrifugation, vacuum-dried, and stored at room temperature.

Somatic polysaccharide fractions were precipitated with five volumes of methanol from the deproteinized supernates previously dialysed against running tap water for 2 consecutive days and condensed to about $\frac{1}{10}$ volume *in vacuo*. The precipitates were collected by centrifugation and vacuum-dried. They were then purified by repeating solution in water, precipitation with methanol, and vacuum-drying. Some of them were further purified by treatment with active carbon powder. They were stored at room temperature. Some of the somatic polysaccharide fractions used were isolated from materials extracted with N NaOH solution and previously deproteinized with trichloroacetic acid. The procedure for isolation was the same as that for the extraction with urea.

Tuberculo-proteins were precipitated at pH 4.2 and 2.2 from Seitz-filtered Sauton culture filtrates condensed to about $\frac{1}{10}$ volume by addition of an adequate amount of diluted hydro-

TABLE I
Chemical and Serological Properties of Antigens

Antigen	Properties	N	Ps	M.-D.	B.	Precipitin test
		<i>per cent</i>	<i>per cent</i>			
Polysaccharides	SI	1.17	40	1280*	80	
	SII	0.72	60	2560	0	10,000‡
	SIII	0.16	81	1280	0	5,000
	S1	0.02	68	0	0	100,000
	S6	2.27	21	0	0	100,000
Proteins	RXI	11.7	12.1	640	640	10,000
	R1	8.7	4.2	1280	2560	10,000
	PPD-S	13.6	3.2	80	320	10,000
	R6	11.5	2.0	20	640	5,000
	RIX	11.9	0.6	0	1280	0
	R7	10.7	1.6	0	0	1,000

N, nitrogen (Micro-Kjeldahl).

Ps, polysaccharide measured as glucose (anthrone).

M.-D., Middlebrook-Dubos test.

B., Boyden test.

* Reciprocals of antibody titers.

‡ Reciprocals of antigen titers to eightfold antiserum (ring method).

chloric acid. They were likewise purified by repeated solution in 0.1 M NaOH solution and precipitation at pH 4.2 and 2.2 with hydrochloric acid. Some of them were further purified by dissolution in 90 per cent phenol and precipitation with five volumes of methanol. This was done to eliminate impurities of polysaccharine nature. They were finally centrifuged, vacuum-dried, and stored at room temperature.

The phosphatide fractions were isolated with methanol from tubercle bacilli previously extracted with cold acetone for 3 consecutive weeks in a percolating apparatus devised by Macheboeuf and Fethke (12). The methanol extraction was done at room temperature for 5 consecutive days in an Erlenmyer flask and under constant agitation by means of a magnetic stirrer. The methanol extracts, after having been Seitz-filtered, were evaporated to dryness. The yellowish materials were then treated with boiling acetone to eliminate hot acetone-soluble fats. The residues were dissolved in chloroform and Seitz-filtered. Acetone was added to the yellowish limpid chloroform solution to give a yellowish white smooth precipitate which was collected by centrifugation and vacuum-dried. The purification was done by repeated solutions in chloroform and precipitation with acetone. The final precipitates were collected by centrifugation, vacuum-dried, and stored at -30°C .

Of the more than 20 fractions tested, several were found to be serologically of almost identical value. For this reason, description will be made in the present paper of the representative 11 fractions of which the analytical data are presented in Table I, except for the phosphatide fraction (Pd. ha)¹ of which the data are shown in the text. The sources and symbols of the fractions are as follows:

¹ Pd. ha, phosphatide fraction.

Polysaccharide fractions:

- SI: extracted with urea from heat-killed and defatted bacilli of the strain H37Rv cultivated on glycerol broth.
- SII: extracted with urea from heat-killed but non-defatted bacilli of the same strain cultivated on the same medium.
- SIII: extracted with urea from acetone-killed bacilli of the same strain cultivated on the Sauton medium.
- S1: extracted with urea from acetone-killed bacilli of the strain Nakano, human type, and purified with active carbon powder.
- S6: extracted with N NaOH solution from acetone-killed mixed bacilli of the strains H37Rv and Nakano cultivated on Sauton medium. Purified with active carbon powder.

Protein fractions:

- RXI: extracted with urea from defatted bacilli of the strain H37Rv on Sauton medium.
- R1: extracted with urea from defatted bacilli of the strain Nakano on the same medium.
- PPD-S: manufactured in the National Institute for Preventive Hygiene, Tokyo. Lot 15.
- R6: tuberculoprotein of BCG, precipitated at pH 4.2.
- RIX: tuberculoprotein of BCG, precipitated at pH 4.2 (another batch), and purified with 90 per cent phenol.
- R7: tuberculoprotein of BCG, precipitated at pH 2.2.

Phosphatide fraction:

- Pd. ha: extracted from acetone-killed mixed bacilli of the strains H37Rv and Aoyama B, human type, cultivated on Sauton medium. Nitrogen, 0.3 per cent, polysaccharide (antrone), 16.9 per cent, and phosphorus, 2.8 per cent.

2. *Antiserum.*—Rabbits, weighing from 2 to 3 kg., received at weekly intervals 3 subcutaneous injections of 1 mg. of heat-killed bacilli of the strain H37Rv, suspended in paraffin oil. 3 weeks after the last injection, the animals were bled and the sera were collected, Seitz-filtered, and stored at 4°C.

3. *Kaolin Suspension.*—In the present study, antigen-sensitized kaolin particles were used with success for absorbing antisera. The method of preparing kaolin suspensions is briefly as follows: Official kaolin powder² is taken in a crucible which is red-heated on a coal gas burner for about 2 hours (ca. 800°C.). The powder, after cooling, is then poured into an agate or glass mortar and pulverized as fine as possible. 2 gm. of the pulverized kaolin is taken into a glass test tube, 15 × 150 mm., to which is added about 10 cm. water. The tube is then shaken vigorously by hand and left to stand overnight at room temperature. The supernate is decanted into another tube or flask. From this supernate 1 mg./ml. kaolin suspension is prepared, distributed by 5 ml. amounts in glass ampoules, sterilized at 100°C for 15 minutes, and stored at room temperature.

4. *Sheep Red Cells.*—One volume of Alsver's solution as recommended by Boyden (1) was added to one volume of defibrinized sheep blood. It was stored at 4°C. For preparing the red cell suspensions, aliquots of the stored blood were filtered through cotton wool and centrifuged for 10 minutes at 2,000 R.P.M. After the supernate was removed, the sedimented red cells were washed 3 times with phosphate-buffered saline at pH 7.2. Blood was used within 3 to 20 days after stored.

5. *Serological Tests.*—

(a) *Middlebrook-Dubos' test:* For preparing the antigen-sensitized red cell suspensions, $\frac{1}{40}$ volume of packed red cells was added to one volume of 0.5 mg./ml. antigen saline solution taken in a flask. The suspension was incubated at 37°C. for 2 hours, during which the flask was shaken every 30 minutes. Then, the sensitized red cells were collected by centrifugation,

² Care must be taken to select a sample of kaolin powder having the proper absorbing power.

washed 3 times with buffered saline at pH 7.2, and finally resuspended in buffered saline to 2.5 per cent. Serial twofold dilutions of sera to be tested, inactivated at 56°C. for 30 minutes, were made with buffered saline in rows of test tubes, 10 × 100 mm., in an amount of 0.5 ml. each and to each, 0.05 ml. of the suspension of sensitized red cells was added. The tubes were incubated at 37°C. for 2 hours. They were shaken every 30 minutes. Readings were made after the tubes were left to stand at room temperature for about 16 hours. The reactions were graded as + + +, + +, +, ±, and -, according to the patterns of the agglutinates formed on the bottoms of the tubes.

(b) *Boyden's test*: One volume of a 20,000-fold dilution of tannic acid was added to one volume of 2.5 per cent red cell suspension in buffered saline at pH 7.2. 10 minutes after the addition of the tannic solution, the red cells were collected by centrifugation at 1,500 r.p.m. for 3 minutes and washed once with buffered saline at pH 6.4. 200 volumes of 0.03 mg./ml. antigen saline solution at pH 6.4 was then added to the tanned red cells. After 10 minutes at room temperature, the red cells were collected by centrifugation and washed once with buffered saline at pH 7.2 to which normal rabbit serum (NRS) was added to a concentration of 0.4 per cent. Finally, the antigen-sensitized red cells were resuspended in NRS-saline to 2.5 per cent. Serial twofold dilutions of sera to be tested, inactivated at 56°C. for 30 minutes, were made in 0.5 ml. amounts with buffered saline at pH 7.2 to which NRS was added to a concentration of 1 per cent. The further procedure was the same as in the case of the Middlebrook-Dubos test, except that readings were made after the reaction tubes had been left at room temperature for 3 hours.

(c) *Phosphatide hemagglutination reaction*: For preparing 0.5 mg./ml. phosphatide saline emulsion to sensitize red cells, one volume of 2 mg./ml. phosphatide methanol solution was added dropwise to four volumes of saline in a beaker and under constant agitation by means of a magnetic stirrer. The beaker was then brought to about 40°C. and the methanol was evaporated by aeration with an electric fan. The emulsion was adjusted with water to the original volume of the saline and used as the sensitizing antigen. The details of the method of the phosphatide hemagglutination reaction have been reported in previous papers (30, 31).

6. Absorption of Antibodies.—

(a) *Procedure with antigen-sensitized red cells*: For absorbing polysaccharide antibodies, one volume of antigen-sensitized sheep red cell suspension, which was made by adding one volume of washed and packed red cells to 20 volumes of 0.5 mg./ml. antigen solution, was added to the same volume of sera to be absorbed, previously inactivated at 56°C. for 30 minutes. The red cell-serum mixture was incubated at 37°C. for 30 minutes, then cooled to 4°C. for 30 minutes, and centrifuged at 2,000 r.p.m. for 5 minutes. An equal volume of the sensitized red cell suspension was again added to the supernate and the same procedure was repeated. Thus, the absorption procedure was repeated 3 times. Tests were performed with the final supernate adjusted to a tenfold dilution of the original serum.

For absorbing protein antibodies, use was made of tanned red cells sensitized with a protein antigen according to the Boyden method (1): five volumes of 2.5 per cent sensitized red cell suspension was added to one volume of inactivated antiserum. The mixture was incubated at 37°C for 30 minutes, then cooled to 4°C. for 30 minutes, and centrifuged at 2,000 r.p.m. for 5 minutes. The same volume of the red cell suspension was added again to the supernate and the same procedure was repeated. The final supernate equal to a tenfold dilution of the original serum was tested.

(b) *Procedure with antigen-sensitized kaolin particles*: The absorption of polysaccharide antibodies was conducted as follows: one volume of 1 mg./ml. kaolin suspension was pipetted in a series of tubes (usually 4 to 5 tubes) containing one volume of 0.1 mg./ml. antigen solution. The tubes were shaken and incubated at 37°C. for 60 minutes, during which time the tubes were shaken by hand every 15 minutes. They were then centrifuged at 3,000 r.p.m. for

5 minutes. The supernates were decanted and the sedimented kaolin particles were washed once with buffered saline at pH 6.8 and centrifuged. The supernates were decanted and the tubes were put upside down on a sheet of filter paper in order to eliminate completely the remainder of the supernate. The inactivated antiserum to be absorbed was then added to 1 of the tubes at about 100 times the volume of the kaolin stuck on the bottom of the tube (*e.g.*, 0.5 ml. serum to 5 mg. kaolin). The tube was then vigorously shaken and incubated at 37°C for 30 minutes. The supernate was thoroughly decanted in the next tube containing antigen-sensitized kaolin pack and the same procedure was repeated. Generally, 3 to 4 repetitions of the procedure were sufficient to eliminate polysaccharide antibodies from the serum.

The same procedure was adopted for absorbing protein and phosphatide antibodies, except that 0.03 mg./ml. antigen solution was used instead of 0.1 mg./ml. solution as for absorbing polysaccharide antibodies. 2 to 3 repetitions of absorption were generally sufficient.³

EXPERIMENTAL RESULTS

1. Hemosensitizing Ability and Chemical Properties of the Antigens.—The results obtained by the M.-D. test and the B. test, using different polysaccharide and protein antigens, are summarized in Table I, along with the nitrogen and polysaccharide contents. As may be seen in the table, the polysaccharide antigens containing less than 1 per cent of nitrogen (SII and SIII) did not sensitize for the B. test. Likewise, the protein antigens containing less than 1 per cent of polysaccharide (RIX) gave no positive reaction for the M.-D. test. It was interesting to see that the ability of the protein antigens to sensitize normal red cells (M.D. test) decreased with their polysaccharide contents, while their ability to sensitize tanned red cells (B. test) was scarcely affected. This fact suggests that, in the case of the M.-D. test, it is the polysaccharide and not the protein that sticks on to the surface of normal red cells, while in the case of the B. test it is the protein and not the polysaccharide that sticks on to the surface of tanned red cells.

Attention must be drawn to the fact that there exist antigens completely deprived of hemosensitizing ability, regardless of their nitrogen or polysaccharide contents (S1, S6, R7), giving, however, positive precipitin reactions, as is shown in the right column of the table. This indicates that hemosensitizing ability and *in vitro* antigenicity are two distinct properties of tubercle polysaccharides and proteins.

Again, it appears from the data that protein antigens containing more than 1 per cent of polysaccharide (RXI, R1, PPD-S, R6) can be of dual character, being capable of sensitizing both normal and tanned red cells to hemagglutination.⁴

³ For the absorption procedure using antigen-coated kaolin, antigens must be as homogeneous as possible, because, in contrast to hemosensitization, adsorption of antigens to kaolin particles is not specific. If the kaolin particles are coated with a protein antigen of dual character as RXI or RI in Table I, they will absorb not only protein antibodies but also polysaccharide antibodies.

⁴ In a recent experiment it was confirmed that, after having been thoroughly deproteinized by the Sevag method (22), several polysaccharide antigens of dual character extracted from

2. *Hemagglutination Tests of Sera Absorbed with Antigen-Sensitized Red Cells.*—Polysaccharide antibodies could be removed from antiserum by absorption with normal red cells previously sensitized with polysaccharide fraction SII, while protein antibodies were removed by absorption with tanned red cells sensitized with protein fraction RIX. The M.-D. and B. tests were performed with these 2 absorbed sera, using the same antigens as the sensitizing antigens, respectively. The results are presented in Table II. As seen in the table, the M.-D. test was negative with the serum absorbed with polysaccharide

TABLE II
Results of Hemagglutination Tests of Sera Absorbed with Antigen-Sensitized Red Cells

Serum	Test	s.d.									
		20	40	80	160	320	640	1280	2560	C ₁	C ₂
Original serum	M.-D. (SII)*	††††	††††	††††	††††	††	+	+	0	0	0
Serum absorbed with SII	M.-D. (SII)	±	0	0	0	0	0	0	0	0	0
	B. (RIX)	+	+	+	+	0	0	0	0	0	0
Original serum	B. (RIX)	+	+	+	+	0	0	0	0	0	0
Serum absorbed with RIX	M.-D. (SII)	††††	††††	††	††	††	††	±	0	0	0
	B. (RIX)	0	0	0	0	0	0	0	0	0	0

C₁, control for red cells; C₂, control for serum.

s.d., serum dilution.

* Symbols are described in the text. M.-D. (SII) = M.-D. test with polysaccharide SII and B. (RIX), B. test with protein RIX.

††††, ††, †: positive reactions graded according to the intensity of reaction; ±, doubtful reaction; 0, no reaction.

SII, while the B. test was unaffected. Inversely, the B. test became completely negative with the serum absorbed with protein RIX, while the antibody titer of the M.-D. test with the same serum was scarcely affected. This fact clearly indicates that the M.-D. test is concerned only with the antigen-antibody system of polysaccharides, while the B. test with that of proteins.

Does the M.-D. test detect only polysaccharide antibodies and the B. test only protein antibodies, when antigens of dual character are used as the sensitizing antigens in both tests?

To answer this question, using normal red cells sensitized with dual protein antigens R1 and PPD-S and polysaccharide SII, respectively, the M.-D. test was conducted with a serum from tubercle bacilli completely lost their ability to sensitize tanned red cells, while their ability to sensitize normal red cells remained almost intact. This fact indicates that the Boyden test using tanned red cells is specifically concerned in the antigen-antibody reaction of proteins.

which polysaccharide antibodies had been previously removed by absorption with polysaccharide SII. (see Table I). At the same time, using tanned red cells sensitized with the same dual protein antigens and protein R6, the B. test was tested with a serum from which protein antibodies had been previously removed by absorption with tanned red cells sensitized with protein R6 (see Table I). The results are presented in Table III.

TABLE III
Results of Hemagglutination Tests of Sera Absorbed with Antigen-Sensitized Red cells

Test	Serum	s.a.	s.d.								C ₁	C ₂
			20	40	80	160	320	640	1280	2560		
M.-D.	Original serum	SII	+++	+++	+++	++	++	+	+	0	0	0
		R1	++	+++	+	+	+	+	0	0	0	
		PPD-S	++	+	+	+	0	0	0	0	0	
	Serum absorbed with SII	SII	0	0	0	0	0	0	0	0		0
		R1	±	±	0	0	0	0	0	0		
		PPD-S	0	0	0	0	0	0	0	0		
	Serum absorbed with R6	SII	+++	+++	++	++	+	+	±	0		0
		R1	++	++	++	++	+	+	±	0		
		PPD-S	++	++	+	0	0	0	0	0		
B.	Original serum	R6	+	+	+	+	+	±	0	0	0	0
		R1	+	+	+	+	+	±	0	0	0	
		PPD-S	+	+	+	+	+	±	0	0	0	
	Serum absorbed with SII	R6	+	+	+	+	+	±	±	0		0
		R1	+	+	+	+	±	0	0	0		
		PPD-S	+	+	+	+	±	0	0	0		
	Serum absorbed with R6	R6	0	0	0	0	0	0	0	0		0
		R1	0	0	0	0	0	0	0	0		
		PPD-S	±	0	0	0	0	0	0	0		

For signs and symbols, see the footnotes of Tables I and II.
s.a., sensitizing antigen.

In the M.-D. test, none of the three kinds of sensitized normal red cells reacted with the serum absorbed with polysaccharide SII, while they all reacted with the serum absorbed with protein R6 almost to the same level as the original non-absorbed serum. Likewise, in the B. test, all the three kinds of sensitized tanned red cells gave no positive reaction with the serum absorbed with protein R6, while with the serum absorbed with polysaccharide SII, they all gave positive reactions as strong as with the original serum.

This fact clearly indicates that, even if dual antigens are used as the sen-

sensitizing antigens, in the case of the M.-D. test it is the polysaccharide moiety of the antigens that sticks specifically on to the surface of normal red cells to react with antiserum, while in the case of the B. test it is only the protein moiety of the antigens that is active. It is clear from these findings that protein itself does not stick to normal red cells. (If protein should stick on to the normal red cell surface, the normal red cells treated with dual proteins R1 and PPD-S would react with the serum absorbed with polysaccharide SII, because protein antibodies remain in the absorbed serum.)

TABLE IV
Results of Hemagglutination Tests of Sera Absorbed with Antigen-Coated Kaolin Particles

Serum	Test	s.d.									C ₁	C ₂
		40	80	160	320	640	1280	2560	5120			
Original serum	M.-D. (SII)	+++	+++	+++	+++	+	±	0	0	0	0	0
Serum absorbed with SII	M.-D. (SII)	0	0	0	0	0	0	0	0	0	0	0
	T. (Pd. ha)	++	++	+	±	0	0	0	0	0	0	0
	B. (R6)	++	++	++	++	+	0	0	0	0	0	0
Original serum	T. (Pd. ha)	+++	+++	++	+	0	0	0	0	0	0	0
Serum absorbed with Pd. ha	M.-D. (SII)	+++	+++	+++	++	+	0	0	0	0	0	0
	T. (Pd. ha)	0	0	0	0	0	0	0	0	0	0	0
	B. (R6)	++	++	++	++	+	+	0	0	0	0	0
Original serum	B. (R6)	++	++	++	++	+	±	0	0	0	0	0
Serum absorbed with R6	M.-D. (SII)	+++	+++	+++	+++	++	±	0	0	0	0	0
	T. (Pd. ha)	++	+	+	±	0	0	0	0	0	0	0
	B. (R6)	±	±	0	0	0	0	0	0	0	0	0

For signs and symbols, see the footnotes of Table II and the text.

T. (Pd. ha) = T. test (phosphatide hemagglutination test) with phosphatide Pd. ha.

3. *Hemagglutination Tests of Sera Absorbed with Antigen-Coated Kaolin Particles.*—Polysaccharide and protein antibodies could also be removed from antiserum by absorption with kaolin particles previously coated with corresponding, relatively simple antigens, polysaccharide SII and proteins R6 and RIX (see Table I). In this experiment, phosphatide antibodies were also easily removed from serum by the use of kaolin particles coated with phosphatide antigen Pd. ha. The M.-D. test using normal red cells sensitized with polysaccharide SII, the B. test using tanned red cells sensitized with protein R6, and the phosphatide hemagglutination test (referred to as T. test in Tables IV and V) using normal red cells sensitized with phosphatide Pd. ha were

conducted with 3 sera absorbed respectively with each of the three different antigens. As seen in Table IV, the results were identical with those obtained in the preceding experiment (see Table II); the M.-D. test was no more positive with the serum absorbed with polysaccharide SII, than was the B. test with the serum absorbed with protein R6. This experiment confirmed that the phosphatide antibody was completely distinct from the other two, antipolysaccharide and antiprotein.

TABLE V
Results of Hemagglutination Tests of Sera Absorbed with Antigen-Coated Kaolin Particles

Serum	Test	s.d.								C ₁	C ₂
		40	80	160	320	640	1280	2560	5120		
Original serum	M.-D. (SII)	+++	+++	+++	++	++	+	0	0	0	0
Serum absorbed with RIX and Pd. ha	M.-D. (SII)	+++	+++	++	+	+	±	0	0		0
	T. (Pd. ha)	0	0	0	0	0	0	0	0		0
	B. (RIX)	0	0	0	0	0	0	0	0		0
Original serum	T. (Pd. ha)	+	+	+	±	0	0	0	0	0	0
Serum absorbed with SII and RIX	M.-D. (SII)	0	0	0	0	0	0	0	0		0
	T. (Pd. ha)	+	+	±	0	0	0	0	0		0
	B. (RIX)	0	0	0	0	0	0	0	0		0
Original serum	B. (RIX)	+	+	+	+	+	±	±	0	0	0
Serum absorbed with SII and Pd. ha	M.-D. (SII)	0	0	0	0	0	0	0	0		0
	T. (Pd. ha)	0	0	0	0	0	0	0	0		0
	B. (RIX)	++	++	+	+	±	0	0	0		0

For signs and symbols, see the footnotes of Tables II and IV and the text.

Further, by absorption with either two of the three different antigens, the sera could be obtained which contained only one kind of antibody reacting only with its corresponding antigen, as demonstrated in Table V. This fact indicates the existence of at least three different kinds of antibodies in tuberculous serum: antipolysaccharide, antiprotein, and antiphosphatide.

DISCUSSION

The present study shows that there exist in tuberculous serum three different kinds of antibodies, antiprotein, antipolysaccharide, and antiphosphatide (Tables IV and V), which can be individually removed from antiserum by absorption with the corresponding antigen. Meynell (13) reported that the

tubercle bacillus had polysaccharide and phosphatide surface antigens and a deep protein antigen, but did not establish that the corresponding antibodies were independent.

Another important fact is that, even if use is made of dual antigens which sensitize both normal and tanned red cells to hemagglutination, the M.-D. test measures specifically polysaccharide antibodies, while the B. test measures specifically protein antibodies (Table IV). This fact clearly indicates that polysaccharide sticks no more on to the surface of tanned sheep red cells than does protein on to the surface of normal red cells. In this connection, Grabar *et al.* (1952) observed that, when a rabbit immune-serum was completely absorbed with normal sheep red cells sensitized with a purified tuberculin polysaccharide containing only 0.4 per cent of nitrogen, the antibody titer of the serum was almost unchanged towards tanned red cells sensitized with PPD. Likewise, when a serum was absorbed with normal red cells sensitized with PPD, its antibody titer remained unchanged towards tanned red cells sensitized with the same antigen. Inversely, when the same serum was absorbed with tanned red cells sensitized with PPD, its antibody titer remained almost unchanged toward normal red cells sensitized with the polysaccharide antigen (6). The results obtained in the present study agree well with these observations.

These findings seem to remove doubt as to the serological specificity of the Middlebrook-Dubos test and the Boyden test, at least so far as tubercle antigens are concerned.

In this connection, Nagai (1960) reported that the active principle for the M.-D. test contained in tuberculin was contained in a polysaccharide fraction precipitable at 50 to 70 per cent methanol. Ordinary protein fractions which have their isoelectric points on the acid side were found to be inactive (16). Tsumita (1960), by chemical analysis, found a lipopolysaccharide of the tubercle bacillus to be the unique active principle for the M.-D. test. (35)

Doubts as to the specificity of the two tests originate from the dual character of tubercle antigens, especially of protein antigens like fractions RXI, R1 and PPD-S as used in the present experiments. As is well known, the so called protein fractions isolated from either tuberculins or tubercle bacilli are of glycoprotein nature and contain generally 2 to 3 per cent of polysaccharide (7). Likewise, polysaccharide fractions so far conventionally isolated from similar materials usually contain traces of nitrogen as seen in the present data (Table I) and in the papers of Hiyama (9), Haworth (8), and Stacey (25). Accordingly, in the light of the results of the present study, most of the tubercle antigens generally used are probably of dual character, and thus can serve as the sensitizing antigens both for the Middlebrook-Dubos test and the Boyden test. Moreover, there has been no convincing evidence as to whether it is really polysaccharide and not protein that sticks on to normal sheep red cells

(3, 11). It is not the procedure itself but the antigen that is to blame for past skepticism of the specificity of the two tests.

As for the inhibition test, it is probable that it gives erratic results because the dual antigens are capable of coupling both polysaccharide and protein antibodies. For example, in the case of the M.-D. test, agglutination of normal red cells sensitized with a polysaccharide antigen will be inhibited to a certain extent by previous addition of a protein antigen to the dilutions of serum, if this is of dual character, and thus it will appear as if the M.-D. test is inhibited by the protein antigen. In this laboratory it has often been found that most protein antigens isolated from tuberculins and tubercle bacilli inhibit to some extent the M.-D. test. The reason for this paradoxical phenomenon is the existence of polysaccharide in the protein antigens. Therefore, the reliability of the inhibition test can be said to depend on the serological homogeneity of the antigens.

Finally, from the results of the absorption tests, it is confirmed that the phosphatide hemagglutination test is also serologically specific as was already pointed out by Takahashi and collaborators (29-31).

SUMMARY

The Middlebrook-Dubos hemagglutination test with normal red cells and the Boyden hemagglutination test with red cells tanned with tannic acid were shown to be strictly specific, for tubercle polysaccharide and for the protein antigens. The former test detects polysaccharide antibodies and the latter protein antibodies. Convincing evidence is given that polysaccharide does not adhere to the surface of tanned red cells nor protein to the normal red cell surface. Hemosensitizing ability and *in vitro* antigenicity were found to be two distinct properties of tubercle polysaccharide and protein antigens.

The phosphatide hemagglutination test with normal red cells sensitized with tubercle phosphatide is also specific; it detects only phosphatide antibodies.

The three different kinds of antibodies, antipolysaccharide, antiprotein, and antiphosphatide, were shown to be completely distinct from each other in tuberculous serum. Each of them can be completely removed from serum by absorption with red cells or kaolin particles coated with its corresponding antigen.

The reliability of the cross-inhibition test is discussed.

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