THE MIDDLEBROOK—DUBOS HAEMAGGLUTINATION TEST AND ITS HAEMOLYTIC MODIFICATION IN THE DIAGNOSIS OF TUBERCULOSIS

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The detection of serum antibodies to *M. tuberculosis* by the agglutination of sheep's red cells previously sensitized with an aqueous extract of phenol-treated bacilli of the H 37/Rv strain was first reported by Middlebrook and Dubos (1948). The short series of tests using human and rabbit sera reported by these authors suggested that the reaction was specific. They showed also that the activity of the antigenic extract was associated with the polysaccharide fraction and that a sample of deglycerinated old tuberculin was equally capable of acting as the sensitizing agent. These findings were confirmed and extended by Gernez-Rieux and Tacquet (1949, 1950a and b) and by Sievers, Ulstrup, and Winblad (1950), using the same methods and antigenic extract as Middlebrook and Dubos.

Many modifications of the test have been described. Scott and Smith (1950) used old tuberculin (Lederle) of four times standard strength as the sensitizing agent, and Rothbard, Dooneief, and Hite (1950), Smith and Scott (1950), Fleming, Runyon, and Cummings (1951), and Kirby, Burnell, and O'Leary (1951) have reported series of tests, using this method. Adcock, Haley, and Davey (1951) and Hinson, Jones, and Chamberlin (1952) used the same antigen, but replaced the sheep cells by human Group O cells. Sohier, Juillard, and Trimberger (1950) used the purified tuberculin prepared by the Pasteur Institute as the sensitizing antigen, and this was compared with the original extract of Middlebrook and Dubos by Gernez-Rieux and Tacquet (1950a and b), who found a close correspondence between the results obtained. Further modifications were described by Sohier, Trimberger, and Juillard (1950) using human Group O Rh-negative cells, the purified tuberculin of the Pasteur Institute as the sensitizing antigen, and a shorter test period. Fewer false positive results were claimed by this technique. Boyden (1951) described a test using Weybridge purified protein derivative (P.P.D.) as the sensitizing agent,

and Hilson and Elek (1951) and Thalhimer and Rowe (1951) both described micro-methods for the test, using human Group O cells.

Haemolytic modifications of the test in which guinea-pig complement was added to the reacting system and lysis occurred in the presence of specific antibody were described by Fisher and Keogh (1950), by Thomas and Mennie (1950), and by Middlebrook (1950). Discrepancies between the results of the haemagglutination and the lysis tests were found both by Middlebrook (1950) and by Mollov and Kott (1952) and the possible existence of two antibodies was postulated.

Considerable differences in the specificity of the tests have been reported both with the various modifications that have been described and where comparable methods have been employed, and the investigation reported here was designed to compare the haemagglutination and lysis tests, to assess the possible clinical value of the results, and to gain some further information as to the effect of varying the conditions and reagents. The material investigated consisted of human sera from healthy subjects, from cases of tuberculosis, and from some patients suffering from diseases other than tuberculosis. The techniques used throughout the investigation required only small quantities of sera, and it was thus possible to perform several tests on each serum.

The Haemagglutination Test

The methods described are essentially those of Middlebrook and Dubos (1948), except for the use of dialysed tuberculin as the sensitizing antigen and of small reagent volumes for the test.

Reagents.—The following reagents were used.

Phosphate-buffered Saline.—Eagle's buffered saline, pH 7.4 (Eagle, 1937), was used throughout. It consists of

NaCl	..	..	..	..	170 g.
KH$_2$PO$_4$	..	..	..	..	2·7 g.
Na$_2$HPO$_4$ (anhydrous)		..	..	11·3 g.
Distilled water to 1 litre.					

The stock solution was autoclaved at 5 lb. pressure for 15 minutes and diluted 1 in 20 with sterile distilled water before use.

Sheep's Blood.—One volume of sheep's blood was collected aseptically into 1.2 volumes of modified Alsever solution, and stored at 4° C. The blood was kept for three days before use and was rejected after one month.

Modified Alsever Solution

Glucose	..	..	..	4·10 g.
Sodium citrate ..	..	..	..	1·60 g.
Sodium chloride	..	..	..	0·84 g.
Distilled water ..	..	..	..	200 ml.

The *p*H was adjusted to 6.1 with 10% citric acid and the solution sterilized in the autoclave at 10 lb. pressure for 10 minutes.

Old Tuberculin.—Burroughs Wellcome old tuberculin was used throughout as the sensitizing agent in testing routine sera. It was deglycerinated, freed from phenol, and rendered isotonic by dialysing in 5-ml. amounts against three 4-litre changes of normal saline over a period of 36 hours. The dialysate was always found to be approximately three times the original volume of old tuberculin. This preparation was stored at −10° C., and under these conditions it maintained its activity for at least a month.

Sera.—Sera were pipetted off on receipt in the laboratory and immediately frozen and maintained at −10° C. Absorbed sera were similarly stored at this temperature.

Test Tubes.—Durham tubes, 6 × 45 mm., were used for the test. They were sterilized in the hot-air oven at 160° C. for one hour.

Methods.—These were as follows.

Absorption of Sera.—Heterophil antibodies to sheep cells were absorbed out by the method of Middlebrook and Dubos (1948). The unabsorbed sera were thawed at room temperature and diluted with an equal volume of phosphate-buffered saline. Complement was inactivated by heating the diluted serum in a water-bath at 56° C. for 30 minutes. To 2 ml. of this inactivated 1-in-2 dilution of serum was added 0.2 ml. of washed packed sheep cells and even suspension ensured by vigorous shaking. The mixture was allowed to stand at room temperature for 10 minutes and the cells then deposited at 2,500 r.p.m. in an angle centrifuge. Without removing the supernatant another 0.2 ml. of packed cells was added and evenly suspended, using a Pasteur pipette, care being taken not to resuspend the deposited cells. After a further 10 minutes at room temperature the cells were spun off in the angle centrifuge and the supernatant removed as in a 1-in-2 dilution of absorbed serum.

Sensitization of Sheep's Cells.—Sheep's cells previously stored in Alsever solution were washed three times in at least 10 volumes of phosphate buffered saline in an angle-head centrifuge. After the last washing the cells were well packed down and as much of the supernatant fluid as possible was removed. One volume of packed cells was added to 49 volumes

of dialysed old tuberculin, well mixed, incubated in a water-bath at 37° C. for two hours, and shaken every 15 minutes. The cells thus sensitized were again washed three times and finally resuspended in phosphate buffered saline to give a concentration of 0.25% for the haemagglutination test. A control suspension of 0.25% unsensitized cells was also made. The suspensions were stored at 4° C. and the sensitized cells were found to be fully agglutinable for about four days, after which time lysis began to be apparent. In practice, however, the cells were sensitized the day before testing the batches of sera.

The Test.—The reagents were added by a dropping technique, using Pasteur pipettes calibrated to deliver 0.02 ml. of water in each drop. The method and volumes (as drops) used in the haemagglutination test are shown in Table I. Serum and antigen con-

TABLE I
THE HAEMAGGLUTINATION TEST

Reagents	Tubes						
	1	2	3	4	5	6 Serum Control	7 Antigen Control
Phosphate-buffered saline ..	2	3	—	2	3	2	4
Serum 1 in 2 ..	2	1	—	—	—	2	—
Serum 1 in 16 ..	—	—	4	2	1	—	—
Sensitized cells ..	4	4	4	4	4	—	4
Unsensitized cells ..	—	—	—	—	—	4	—
Serum dilution* ..	1/4	1/8	1/16	1/32	1/64	1/4	—

* Titres throughout this paper refer to initial serum dilutions and discount the added cell suspensions.

trols were included and a positive serum of known titre and a negative serum were put up with each batch of tests. The contents of the tubes were well mixed by shaking and the tests incubated at 37° C. in the air incubator for two hours. The tubes were then again shaken vigorously to resuspend deposited cells and allowed to stand overnight at room temperature.

Reading the Tests.—The tests were read by gently tapping the tubes with the forefinger until the deposited cells were resuspended. Where agglutination had taken place the cell clumps were disturbed readily, often in a single mass, and the end-point was taken as the last tube showing definite agglutinated clumps as seen by the naked eye. Continued agitation of positive tests readily breaks up the agglutination, and care should be taken to tap the tubes only sufficiently to disturb the deposit. Tests were judged as negative when the deposited cells resuspended less readily on tapping and no agglutinated clumps were seen. Attempts to read the tests according to deposit patterns were abandoned, as it was soon found that agglutinated cells occasionally sedimented as a sharply defined button of cells indistinguishable from the deposit in negative and control tubes.

Repeated tests on the control positive serum and on some test sera showed the method to give results

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which were reproducible to within one tube of the original reading.

The Lysis Test

The preparation of sera and the sensitization of the cells were carried out in the same way as for the haemagglutination test, a 0.5% suspension of sensitized cells being used in place of the 0.25% suspension. The same reagents and test tubes were used.

Preparation of Complement.—Lyophilized guinea-pig complement (Sharp and Dohme) was reconstituted and diluted 1 in 3 with phosphate-buffered saline. One part of washed packed sheep's red cells was added to 15 parts of diluted complement. The mixture was allowed to stand for 10 minutes at room temperature and the cells deposited at 2,500 r.p.m. in the angle centrifuge. The procedure was repeated without removing the supernatant, care being taken not to disturb the deposited cells. The supernatant was finally removed as a 1-in-3 dilution of absorbed complement. It was used within two or three hours and kept meanwhile at 4° C. This method is identical with that described by Middlebrook (1950), except that absorption was carried out at room and not at refrigerator temperature.

Complement must be of high titre. The amount used in this test represents an excess, and a check on the activity of the complement is given by the haemolytic titre of the standard positive control serum included with all batches of tests. Consistently reproducible results were obtained in practice.

The Test.—The tests were set up by the dropping technique. The method and volumes were the same as for the haemagglutination test except that one drop of the absorbed complement was added to each tube. Similar controls were also included. The racks were placed in an air incubator at 37° C. for one hour and were well shaken both before incubation and at 15-minute intervals. On removal from the incubator the tests were read and an arbitrarily judged 50% haemolysis was taken as the end-point. There was no difficulty in determining the haemolytic titres, as in all cases the end-point was sharply defined and in the vast majority of positive tests haemolysis was complete in all but the last tube. As with the haemagglutination test the results were found to be reproducible to within one tube of the original reading. After storage for several months at −10° C., however, some sera showed a definite reduction of lytic activity.

Effects of Modifying the Reagents and Conditions of the Tests

The Red Cells.—Sheep's blood preserved in Alsever solution was compared with defibrinated sheep's blood for the haemagglutination test. The degree of agglutination was most marked using " Alsever " cells and the titres were usually one tube higher than with defibrinated blood. In comparative experiments cells from defibrinated blood were found to be as effective as those from blood in Alsever solution for absorbing the heterophil antibodies from the sera.

Comparisons were made for both the haemagglutination and lysis tests, using sheep cells and human Group O Rh-negative cells. The human blood was preserved in Alsever solution or in the acid-citrate-dextrose solution used for preserving blood for transfusion (Mollison, 1951). The conditions of washing and sensitizing the human cells were those described for sheep cells. The sera used for comparative studies were divided into two parts. Both parts were diluted 1 in 2 and inactivated at 56° C. for 30 minutes, but only the portion used for testing with sheep cells was absorbed. Comparative tests were performed simultaneously on 14 sera known to contain haemagglutinins. One serum gave the same titre (1 in 128) with both types of cell, but the others agglutinated sheep cells to titres which were between two and 16 times higher than those obtained with human cells. Thus, not only were human cells less agglutinable than sheep cells, but the discrepancies between the results were variable and could not be resolved by the use of a conversion factor. Similar discrepancies were noted when the tests were read by the deposit pattern. One serum from a non-tuberculous subject which was known to contain haemagglutinins was also tested and gave identical titres (1 in 16) with both human and sheep cells.

Sensitized human cells were valueless for the lysis test by the technique used, as lysis occurred with only one of the 14 sera and in this case the lysis titre was 64 times lower than when sheep cells were used. Ten of the 14 sera gave lysis of sensitized sheep cells at titres of 1 in 8 or over.

The Antigen.—Comparisons were made of four sensitizing antigens : dialysed old tuberculin (Burroughs Wellcome), dialysed old tuberculin (Evans), P.P.D. (Weybridge) 2.5 mg. per ml., and the crude polysaccharide fraction of old tuberculin described by Hilson and Elek (1951). In the comparative tests all serum dilutions were made in bulk and then distributed into duplicate series of tubes.

Six sera were tested by the haemagglutination technique using sensitizing antigens prepared by dialysis of Burroughs Wellcome's and of Evans' old tuberculin. The titres in both cases were found to be identical.

Hilson and Elek (1951) claimed greater specificity for an antigen derived from the partial fractionation of old tuberculin and described as the crude carbohydrate fraction or C.C.F. This

was prepared according to their directions, and a comparative series of tests on four sera from tuberculous and five from non-tuberculous subjects were put up by the haemagglutination and lysis methods, using cells sensitized with old tuberculin and with C.C.F. All these sera were known to contain haemagglutinins and four gave positive lysis tests. The agglutination of cells sensitized with C.C.F. occurred more rapidly and was somewhat more intense than with tuberculin-sensitized cells, but no significant difference was found between the final titres for either the haemagglutination or the lysis tests and the use of C.C.F. gave no evidence of increased specificity.

Purified protein derivative (Weybridge) was used by Boyden (1951) as a sensitizing agent both for a modification of the Middlebrook-Dubos test and for a test he described for the detection of antibodies to tuberculo-protein in which the cells were previously treated with tannic acid. Seventeen sera were tested by the haemagglutination method to compare the sensitizing effect of undiluted dialysed tuberculin with that of an equal volume of 1 in 10 P.P.D., 2.5 mg. per ml. Identical titres were obtained in all cases. Five further sera were used to compare 1 in 10 P.P.D. with a 1 in 4 dilution. Both concentrations were equally effective. Purified protein derivative, 1 in 10, will sensitize sheep cells for the lysis test, although titres were somewhat lower than when dialysed old tuberculin was used. Higher concentrations of P.P.D. were not tried.

Experiments comparing the specific inhibition of the haemagglutination reaction by P.P.D. and by a purified tuberculopolysaccharide indicated that the fraction of P.P.D. responsible for sensitizing the cells was the 5% of contaminating polysaccharide present in the preparation.

Sensitization of the Cells.—The effect of varying three of the conditions of sensitization was studied.

Effect of Alteration of pH.—Dialysed tuberculin was mixed with equal volumes of 0.1M phosphate buffer solution and three batches of cells washed in unbuffered saline were sensitized at pH 6.4, 7.0, and 7.6. Haemagglutination titres and the degree of agglutination were identical when these batches were tested with a known positive serum.

Effect of Diluting the Antigen.—Sheep cells were sensitized with dialysed old tuberculin in concentrations of 1 in 1, 1 in 2, and 1 in 4. The suspensions were then tested against a known positive serum. The degree of agglutination and the end-titres were identical with the undiluted and 1 in 2 dilutions of the sensitizing antigen,

although agglutination was more apparent after two hours at 37° C. with the undiluted material. Dilution of the tuberculin to 1 in 4 resulted in a diminution of titre of one tube and less marked agglutination. The antigen was therefore used undiluted for all tests.

Effect of Variations in Temperature of Sensitization.—Cells treated with dialysed old tuberculin for two hours at 37° C., 20° C., and 4° C. were compared by haemagglutination with a known positive serum. Sensitization was more complete at 37° C. than at 20° C., and there was no agglutination of cells treated at 4° C.

Effect of Variations in Size of Tube for Haemagglutination Test and in Quantities of Reagents.—Haemagglutination tests were performed in tubes of 12 mm., 9 mm., and 6 mm. diameter respectively, using varying volumes of reagents. As in previous tests, the serum dilutions were made in bulk and subsequently distributed to the test racks in order to reduce any inherent technical errors. There was no loss of sensitivity in using 6 mm. tubes, and a total volume of 0.16 ml. was found to be most easily read for the haemagglutination tests and gave identical titres to those obtained with 0.8 ml. amounts in the larger tubes.

Effect of Varying Period and Temperature of the Test.—During the early stages of the investigation discrepancies were noticed between readings of the tests made after two hours at 37° C. and after standing overnight at room temperature. Some positive sera agglutinated to titre after two hours at 37° C., while others showed little or no agglutination at this time, but when left at room temperature overnight agglutination became apparent.

Thirteen sera from tuberculous and non-tuberculous subjects containing haemagglutinins were tested against sensitized sheep cells at different temperatures for 16 hours. The tubes were well shaken at two and four hours, as, in the tests incubated in the water-bath at 37° C., the cells sedimented more rapidly, and it was considered desirable to keep all the cells in suspension for an approximately comparable time. The results are shown in Table II.

Of the seven sera from non-tuberculous cases five gave higher titres at room temperature than at 37° C., while the other two showed no change. Of the six sera from tuberculous cases two showed higher haemagglutinin titres at 37° C. than at room temperature, three gave identical titres, and one showed greater activity at room temperature. No absolute distinction between

TABLE II

EFFECT OF VARYING THE TEMPERATURE FOR
HAEMAGGLUTINATION TEST

	Serum No.	Serum Titres		
		37° C.	Room Temperature	4° C.
Non-tuberculous sera	34	1/8	1/16	—
	93	<1/2	1/4	—
	92	1/2	1/16	—
	32	1/4	1/4	1/8
	36	1/16	1/16	1/8
	47	1/4	1/8	1/8
	76	<1/4	1/8	1/8
Tuberculous sera	104	1/128	1/32	1/32
	81	1/128	1/64	—
	99	1/32	1/32	1/16
	86	1/64	1/64	1/64
	82	1/16	1/16	1/16
	103	1/16	1/32	1/16

haemagglutinins in tuberculous and non-tuber-culous sera was detected by this method, but these preliminary observations suggest that there is a tendency for them to have different optimum temperatures of activity.

The Series and Methods of Investigation

The main purpose of this section of the investigation was to compare the haemagglutination and lysis tests and to determine which of them, if either, would merit an extended investigation of its possible use as a diagnostic test.

The sera investigated comprised a selected group drawn mainly from cases of active tuberculosis and from presumably healthy subjects. The few sera from cases of other diseases and from doubtfully tuberculous patients were tested at a time when a clinical diagnosis of tuberculosis was considered probable. Sera were obtained from 150 subjects in all and their origin is shown in Table III.

TABLE III

ORIGIN OF SERA EXAMINED BY COMPARATIVE
HAEMAGGLUTINATION AND LYSIS TESTS

Category	No. of Sera
Healthy blood donors	69
Healthy antenatal patients	15
Other healthy subjects	11
Patients suffering from diseases other than tuber-culosis	3
Cases of pulmonary tuberculosis	42
Cases of pelvic tuberculosis	1
Cases of tuberculous meningitis	7
? Tuberculous cases	2
Total ..	150

Of the 11 sera taken from the group classified as "other healthy subjects," two had been inoculated with the vole bacillus six months previously and eight were tuberculin-negative. All but one of these 11 cases had been previously tuberculin-

tested. Of the three patients suffering from diseases other than tuberculosis, one was bronchiectatic, one had a virus meningitis, and one was an apparently cured case of sarcoidosis whose tuberculin reaction had recently converted. Two doubtfully tuberculous cases were included in the series. In both of these the diagnosis was considered in view of a suspicious radiological opacity, but clinically no evidence of active tuberculosis was found.

These 150 sera were tested in groups of about 40, and haemagglutination and lysis tests put up simultaneously, using the same batches of sensitized cells. The controls already described were included for all sera, and a positive and negative serum of known titre was put up with each batch of tests as a check on the sensitization of the cells. At the time of reading the tests the sera were identified only by a serial number, and it was not known from which type of case they had originated. Sera giving positive reactions throughout the dilutions of the test were subsequently retested at higher dilutions to determine the endpoint.

The results of tests on the 50 tuberculous and 98 non-tuberculous cases are shown as histograms in Fig. 1.

Most workers have considered titres of 1 in 8 or of 1 in 16 as positive for the haemagglutination test, and in this series a " positive " titre of 1 in 16 or over gives the best separation of results. For the lysis test Middlebrook (1950) considered a titre of 1 in 8 or over as positive, and these results confirm that this is the optimum point at which to place an arbitrary diagnostic level.

When comparisons between the two tests are made with these titres as the dividing line between positive and negative results, 12 of the 98 non-tuberculous cases give false positive results by the haemagglutination method compared with six by the lysis method, and 18 of the 50 tuberculous cases are " negative " by the haemagglutination test as against 16 by the lysis test. As the two-fold dilution techniques used in testing the sera have an inherent error of one tube, it is of importance to compare the likelihood of such an error changing the interpretation of an individual test. Taking the " diagnostic " level at the titres indicated above, 49 of the results of the haemagglutination tests lie on either side of this arbitrary dividing line compared with only 20 of those of the lysis tests. The lysis test thus shows not only a higher proportion of positive results in cases of tuberculosis and fewer false positives in non-tuberculous cases, but also a greater separation of results on either side of the diagnostic level.

Sera from Cases of Pulmonary Tuberculosis.—Sera from 42 cases of pulmonary tuberculosis were tested. Thirty of these were positive by the lysis test and 29 by the haemagglutination test. Three sera gave positive haemagglutination but negative lysis tests; in one the haemagglutination titre was 1 in 128 and the lysis titre <1 in 4, a finding which was confirmed in five samples of serum from this patient received over a period of three months. Four cases showed positive lysis tests and negative haemagglutination tests. One serum gave a haemagglutination titre of 1 in 16 only and a lysis titre of 1 in 128, and it is of interest that a positive Coombs reaction was obtained on tuberculin-sensitized cells which had been incubated for 30 minutes at 37° C. with a 1 in 50 dilution of this serum.

Information regarding the clinical condition of the patients, of the Ministry of Health grade, and the result of the last E.S.R. was sent with each specimen. In some cases the information was incomplete, but the clinician's opinion as to the clinical activity of the lesion at the time of taking the specimen and the grade to which the patient's lesion was allotted are shown in Table IV and compared with the results of the tests. The figures, though small, suggest a positive correlation between the serological results and the activity of the
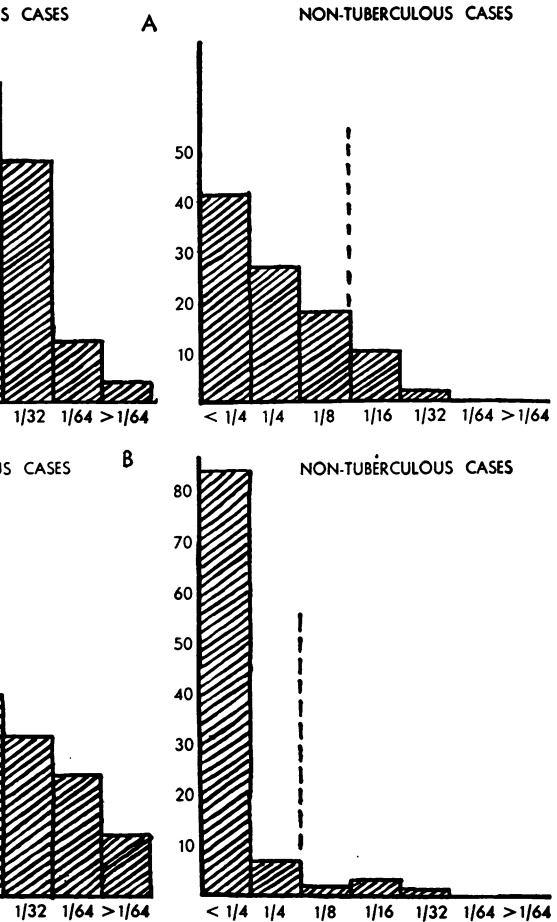

FIG. 1.—A, results of the haemagglutination test on 148 sera; B, results of the lysis test on 148 sera.

disease. A less subjective comparison is shown as a spot diagram in Fig. 2, in which E.S.R. results from cases of pulmonary or meningeal tuberculosis are compared with the haemagglutination or lysis titres of sera taken at approximately the same time. These results indicate a well-marked correlation between the results of the E.S.R. and

TABLE IVA

CORRELATION BETWEEN RESULTS OF HAEMAGGLUTINATION AND LYSIS TESTS AND MINISTRY OF HEALTH GRADE IN 40 CASES OF PULMONARY TUBERCULOSIS

Ministry of Health Grade	Haemagglutination Test		Lysis Test	
	Positive	Negative	Positive	Negative
Grade A	2	—	—	2
Grade B1	2	2	2	2
Grade B2	18	10	20	8
Grade B3	5	1	6	—

TABLE IVB

CORRELATION BETWEEN CLINICAL ACTIVITY OF THE LESION IN 41 CASES OF PULMONARY TUBERCULOSIS AND THE RESULTS OF THE SEROLOGICAL TESTS

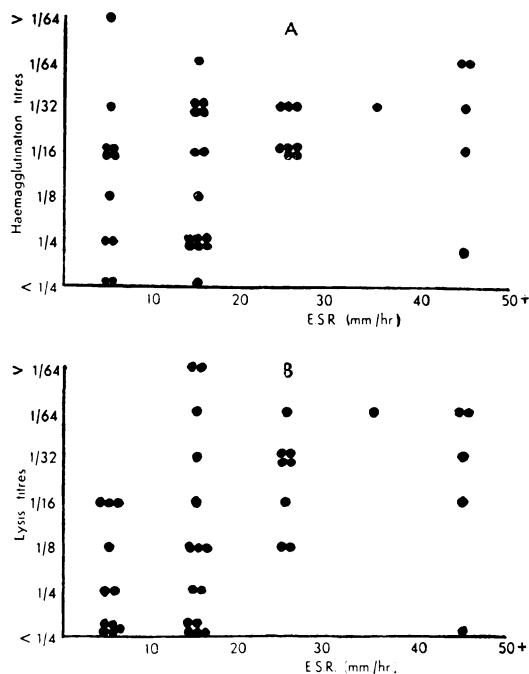| Clinical Activity	Haemagglutination Test		Lysis Test	
	Positive	Negative	Positive	Negative
Quiescent	2	2	2	2
Regressing	12	6	12	6
Stationary ..	6	0	5	1
Progressing ..	10	3	12	1

FIG. 2.—A, correlation between haemagglutination titres and E.S.R. results; B, correlation between lysis titres and E.S.R. results.

of the serological tests and particularly of the lysis test. Special mention should be made of the point on this diagram referring to an E.S.R. of between 40 and 50 and a haemagglutination titre of 1 in 4 and lysis titre of less than 1 in 4. This patient had a gross, superadded non-tuberculous pulmonary infection and there was considerable doubt as to the activity of the tuberculous lesion.

All but 10 of these cases had been treated with streptomycin or combined streptomycin and P.A.S. Eight of these untreated cases gave positive haemagglutination tests with titres of up to 1 in 32, and eight gave positive lysis tests with titres up to 1 in 128. This small group is a reasonably representative sample of the whole series.

Sera from Cases Presenting Primarily as Tuberculous Meningitis.—The sera from seven cases of tuberculous meningitis were tested. Three were positive by both tests and four negative. Two of those giving positive results were clinically active and were suffering from pulmonary tuberculosis in addition to the meningeal lesions, and both patients died. The other patient from whom a positive result was obtained was a case of uncomplicated tuberculous meningitis whose disease was clinically considered to be arrested; her E.S.R., however, was 59 mm. in one hour. She was subsequently discharged as cured. Of the four

patients giving negative serological tests the disease was definitely arrested in three and probably in the fourth ; one of these patients had previously suffered from pelvic tuberculosis which was treated surgically, and one had an inactive pulmonary lesion treated by artificial pneumothorax. They were all subsequently discharged as cured.

Sera from Blood Donors.—Sixty-nine specimens of sera were received from healthy blood donors. Sixty of these were negative when tested by the haemagglutination method and 65 were negative by the lysis test. Six sera gave a positive haemagglutination test and negative lysis test ; in four of these the lysis titre was less than 1 in 4, and in the other two lysis was apparent to a titre of 1 in 4 only. One serum gave haemagglutination to a titre of 1 in 8 (negative) and lysis to a titre of 1 in 16.

Three of these sera were thus positive by both the haemagglutination and lysis tests, and inquiries as to the clinical condition of these subjects revealed no evidence to suggest that they were tuberculous, but it was not possible to subject them to a full physical and radiological examination.

Sera from Antenatal Patients.—Fifteen sera from healthy antenatal patients were tested. Fourteen of these were negative by both the haemagglutination and lysis tests and one was positive by both methods ; no evidence of tuberculosis or other illness was found in this case at routine antenatal examination, but it has not been possible to re-examine her physically or radiologically.

Sera from Other Non-tuberculous Healthy Subjects.—Sera were tested from 11 other healthy non-tuberculous subjects. All but one of these had previously been tuberculin-tested and two had been inoculated with the vole bacillus vaccine eight months previously. Two of the 11 sera gave positive haemagglutination tests, and all were negative by the lysis test. The relevant data on the 10 patients who had been tuberculin-tested is summarized in Table V. These results give no direct support to the view that tuberculin-testing *per se* may give rise to haemagglutinins or lysins to tuberculin-sensitized cells. Further evidence on this point is presented below.

Sera from Cases of Non-tuberculous Disease.—Sera were tested from three patients only. One was suffering from mumps encephalitis and gave negative results by both the haemagglutination and lysis tests ; one had a collapsed and bronchiectatic left lung and similarly gave negative sero-

logical results. The third was a case of apparently healed pulmonary sarcoidosis. This patient was in good health, and had a normal E.S.R. and negative radiograph, but the Mantoux test had converted to positive within the previous six months. The haemagglutination test was negative but the lysis test positive in this case (HA. 1/8, L. 1/16). This subject was a laboratory worker and exposed to infection by tubercle bacilli.

Sera from Cases of Suspected Tuberculosis.—
Sera were tested from two cases with suspicious radiological lesions. Both were clinically in good health and have remained so. One gave a positive haemagglutination and negative lysis test (HA. 1/16, L <1/4) and the other was negative by both tests (HA. 1/4, L. <1/4).

Effect on the Haemagglutination and Lysis Tests of Previous Tuberculin Injections

The results of tests on the sera of 10 normal subjects are summarized in Table V and have

TABLE V

SEROLOGICAL FINDINGS ON 10 SERA FROM
SUBJECTS PREVIOUSLY TUBERCULIN-TESTED

Serum No.	Previous Vole Vaccine	Months since Last Tuberculin Test	Mantoux Result	Titre	
				Haemagglutination Test	Lysis Test
55	No	5	+ve	1/8	<1/4
56	Yes	5	+ve	1/16	<1/4
57	,,	8	+ve	1/4	<1/4
105	No	1	−ve	<1/4	<1/4
106	,,	1	−ve	<1/4	<1/4
107	,,	1	−ve	1/16	<1/4
108	,,	1	−ve	1/4	<1/4
114	,,	1	−ve	1/8	<1/4
115	,,	1	−ve	<1/4	<1/4
116	,,	1	−ve	1/4	<1/4

already been referred to. When these results are compared with those in Fig. 1 for all non-tuberculous subjects they may be seen to be a representative sample. Serial tests were also made on the sera of three non-tuberculous, tuberculin-positive subjects following the intrathecal inoculation of 0.00375 mg. of P.P.D. The sera were taken both before the inoculation and one and five days afterwards ; in one case a further test was made seven days after the injection. No rise of haemagglutinin or lysin titres occurred in these cases.

More significant evidence of the failure of tuberculin inoculations to cause the development of lysins was obtained by the repeated examination of sera from patients under treatment with streptomycin and intramuscular tuberculin inoculations. Six such cases were studied, and all the specimens

of sera from each case were tested together under standard conditions.

F.B., aged 45, is cited as an example. Nine sera were taken over a period of two months during which a full course of tuberculin and streptomycin treatment was given. This patient had extensive fibrosis, bronchiectasis, and emphysema, and it was subsequently believed that the principal factor in his infection was non-tuberculous. The results are summarized below :

Date	Tuberculin Dose (ml.)	Haemagglutination Titre	Lysis Titre
27.12.51	0·002	1/4	<1/4
15.1.52	0·06	1/8	1/4
28.1.52	0·1	1/4	<1/4
2.2.52	—	<1/4	<1/4
7.2.52	0·2	<1/4	<1/4
15.2.52	0·4	<1/4	<1/4
21.2.52	0·6	1/4	<1/4
22.2.52	—	1/4	<1/4
27.2.52	—	1/8	<1/4

These results, together with those from the other five patients, indicate that some slight rise in haemagglutinin titre may follow the intramuscular inoculation of high doses of tuberculin ; this is, however, an irregular effect. The lysis titre is not significantly affected by injections of large doses of tuberculin.

Discussion

Numerous serological tests for antibodies to *M. tuberculosis* have been described (Wilson and Miles, 1946), but none has yet been generally accepted as a practical advance in the diagnosis of the disease. The haemagglutination test of Middlebrook and Dubos (1948) promised to give better results than those used previously, and Gernez-Rieux and Tacquet (1950b) in a comparative series demonstrated the superiority of the haemagglutination test over complement-fixation tests, using both the Besredka egg antigen and the Nègre and Boquet antigen. In almost all reported series of haemagglutination tests, however, there have been a proportion of false positive results, and the findings described in this paper are very similar to those obtained with similar techniques by Fleming *et al.* (1951), Kirby *et al.* (1951), and Mollov and Kott (1952), in which haemagglutinins were detected in the sera of about half the non-tuberculous subjects investigated and agglutination occurred to " diagnostic " titre in about 10% of these cases.

It is clear that if the haemagglutination test is to be of practical diagnostic value some means must be found to increase its specificity. The use of human Group O cells decreases the sensitivity

of the test markedly and in an irregular fashion, and although it eliminates the necessity of preliminary absorption of the sera the results are less satisfactory than with sheep cells: variation of the period and temperature of incubation also seems to offer little prospect of any considerable improvement of the test. Some workers have claimed greater specificity with the use of partially purified fractions of tuberculin, and although no difference in the agglutination titres was found in comparative experiments with four different antigens in this investigation, it seems possible from the work of Pound (1952) that further fractionation of the polysaccharide antigens may improve the results.

The lytic modification of the test shows greater promise as a useful method for the diagnosis of tuberculosis, and the comparative series of the two tests reported here confirm the findings of Middlebrook (1950) and Mollov and Kott (1952) that it is more specific for the disease. The discrepancies between the haemagglutination and lysis titres on individual sera also support the view of Middlebrook (1950) that a different antibody is responsible for the reaction, and the lysis test certainly merits further study in view of its superiority to the haemagglutination method.

In any future assessment of the haemolytic modification the method should include a complement titration and a more accurate dilution technique covering a closer range of dilutions than those used here. One such method has been described (Maillard and Gagliardo, 1951 ; Maillard, 1952), and has apparently proved satisfactory in practice.

Some investigations have been reported of the relationship between haemagglutinin titres and the extent, severity, and prognosis of the tuberculous disease (Gernez-Rieux and Tacquet, 1950b ; Sohier, Juillard, and Trimberger, 1950 ; Spain, Childress, and Rowe, 1952 ; Hinson et al., 1952), but much less work has been done on this aspect of the subject with the lytic modification of the test. The results of this series suggest that the lysis titre is related, in part at least, to the activity of the lesion, but the cases were not followed up for long enough to show what other factors may be involved. It will be of great interest to determine whether these antibodies are related in any way to the development of immunity in tuberculosis ; but any such study in human cases presents considerable difficulties, as the response to the infection is almost invariably modified by the therapeutic measures employed.

The development of antibodies to tuberculin-sensitized cells following intradermal tuberculin inoculations was described by Smith and Scott (1950) and by Gerstl, Kirsh, Andros, Winter, and Kidder (1952) and would, if confirmed, have to be taken into account in the interpretation of the test. In the cases reported here, however, neither large intramuscular doses of tuberculin nor previous Mantoux testing affected the lysis test, although a small rise in the haemagglutinin titres was sometimes detected. There is thus no evidence that previous tuberculin inoculations will give rise to false positive lysis tests if the technique described here is used.

In conclusion, the results of this investigation show that the test for lysis of tuberculin-sensitized sheep cells in the presence of antibody and complement is more specific for tuberculosis than the haemagglutination test, and it is considered that further extended studies of this method should be carried out to assess its value as a diagnostic and prognostic test. It will be important, however, in any such investigation to be able to subject healthy and other presumably non-tuberculous subjects to full radiological and clinical investigation if necessary in addition to studying repeated samples of serum from tuberculous patients throughout the course of their disease.

Summary

The Middlebrook-Dubos haemagglutination test for tuberculosis was compared with its lytic modification ; the lysis method was shown to be more specific as a diagnostic test.

Marked discrepancies between the two tests performed on individual sera were found and evidence was presented to support the postulate that different antibodies are concerned.

Experiments were undertaken to determine the effect of varying some of the conditions and reagents for the haemagglutination test. Sheep cells were shown to be superior to human cells, and alterations in the duration and temperature of incubation caused wide variations of the final titre. Human cells were found to be useless for the lysis test by the technique described.

Four sensitizing antigens were compared for the haemagglutination test and no significant difference was detected between them.

Massive doses of intramuscular tuberculin were shown to have little effect on the results of the haemagglutination test and none on the lytic modification.

The haemagglutination test described was considered to be of little value in the diagnosis of tuberculosis, but the lytic modification should be further investigated.

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