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Further studies on the serologic diagnosis of the rheumatoid factor : a comparison of the dextran procedure against the standard serum latex and euglobulin procedure

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FURTHER STUDIES ON THE SEROLOGIC DIAGNOSIS OF THE RHEUMATOID FACTOR - A Comparison of the Dextran Procedure against the Standard Serum Latex and Euglobulin Procedure

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Since the observation by Cecil et al (1) in 1930 that serum from patients with rheumatoid arthritis would agglutinate certain strains of streptococci, and by Waaler (2), in 1940, that such sera would also agglutinate sheep cells sensitized with rabbit anti-sheep-cell serum, it has become clear that sera from rheumatoid arthritis patients contain a factor, or factors, that is either absent or present in minimal amounts in the sera of normal patients. The presence of such a factor has been demonstrated by a variety of serologic indicator systems such as paper chromatography (3), ion-exchange chromatography (4), and the ultra-centrifuge (5). Regardless of these many methods of demonstrating the existence of a rheumatoid factor, the exact nature of this factor remains unknown. (It will not be the intent of this paper to make further attempt at its identity.)

The fact that the so-called "rheumatoid factor" appears quite consistently in the sera of patients with rheumatoid arthritis has led to a succession of serologic reactions in an effort to lend laboratory diagnosis of a

"practical nature" to the practicing clinical physician.

The first tests described were based upon a precipitin reaction occurring with a large particle, the erythrocyte. The erythrocyte has a complex organic structure, which leads to difficulty in the procedure and uncertainties in interpretation of the reaction. The sheep erythrocytes used contain many antigens which react with components of human and animal sera. As erythrocytes from many animals were used, a constant titer could not be achieved. Cells from the same animal could not be stored and maintain a constant titer. Thus the first diagnostic test for the rheumatoid factor, devised by Rose et al (6), entertained many variables.

Heller (7) modified this test by using tannic acid suspensions of sheep erythrocytes mixed with pooled human plasma fraction II. This technique was very laborious and the complexity of the organic and antigenic structure of the erythrocyte was not thereby eliminated. These tests still did not measure up to desired accuracy and many factors were left unexplained.

In seeking to explain the high percentage of negative agglutination tests in patients with rheumatoid arthritis, the possible presence of an inhibitor substance was considered. A variety of sera were thus tested and inhibition was achieved with a high percentage

(37%) of non-rheumatoid sera. This suggested the possibility of increasing the sensitivity of the agglutination reaction by separation of the inhibitor substance from the agglutination factor. Partial separation was achieved by dialysis of the euglobulin fraction at pH 6.0 against a phosphate buffer. Testing of this euglobulin fraction gave 92% positive agglutination of rheumatoid sera with only 2% false positives in normal controls.

When the capacity of the euglobulin fraction to inhibit agglutination of known positive rheumatoid serum was investigated, it was found that 100% of rheumatoid sera failed to inhibit, whereas only 5% of control sera behaved in such a manner (8).

These results indicated that measurement of either the agglutinating or inhibiting activity of the euglobulin fraction provided a basis for a sensitive test. However, at that time (1954) the complex nature of the sheep red cell still left many variables which were not able to be adequately controlled.

Singer and Plotz (9) proved that biologically inert polyvinyl latex particles could be substituted for sheep cells in investigating rheumatoid arthritis sera. They conducted a series of experiments, using latex particles, to set up standard conditions for which the interaction between gamma globulin and rheumatoid arthritis

sera could best be applied as a diagnostic test for the rheumatoid factor. These experiments concluded: (1) particles of 0.77 to 0.98 microns were satisfactory. A stock solution of 1:100 corresponds to a turbidity of 6% light transmission which was optimum concentration. (2) Borate buffer between pH 8.2 and 9.0 proved most suitable for the reaction. (3) Addition of 0.85% NaCl solution to the system results in a marked increase in agglutination. (4) The optimal concentration of gamma globulin was 250 gamma per ml., giving a final dilution in test sera of 125 gamma per ml.. (5) Optimum agglutination occurred after inactivation for two hours at 56 degrees centigrade. These conditions have since become standards for any procedure utilizing latex particles. However, work is still being carried forward to improve the accuracy of the test by improving any of the standard conditions.

The latex fixation reaction has provided us with a relatively simple, rapid and direct method of testing the interaction between rheumatoid serum and fraction II globulin. A series by Plotz and Singer (10), provided only 71% positive results with known rheumatoid sera. This series also resulted in positive results with 1% of normal sera, 3% of patients with non-arthritic diseases, 5% of patients with hyperglobulinemia, 2% of osteoarthritic

1.6% of patients with rheumatic fever and 5% of patients with disseminated lupus erythematosus (Table I).

Efforts have thus been turned in the direction of developing reactions which will eliminate false positives and increase sensitivity and specificity.

As a result of reports by Ziff et al (8) of the increased sensitivity with use of the euglobulin fraction, the euglobulin methods have been combined with latex fixation methods (11). The sensitivity was greatly improved by this technique without increasing false positives.

Sensitivity has been the highest with euglobulin inhibition techniques. However, this test is too technical and laborious to be considered practical in most diagnostic laboratories unless they are of considerable size.

One of the newest additions to the multiple serologic tests for the rheumatoid factor is a procedure utilizing a dextran solution for washing human red cells (12), which will be described in detail later in this paper. The abnormal protein coating the erythrocytes of rheumatoid patients can not be removed with saline washing.

The results of the original article (12) of using the dextran-obtained protein in the standard latex-fixation

TABLE I

RESULTS OF LATEX FIXATION TEST IN PATIENTS WITH RHEUMATOID ARTHRITIS AND OTHER CONDITIONS

Clinical Groups	Total Cases	Positive	Percentage
Rheumatoid Arthritis	150	107	71.3
Osteoarthritis and other arthritis	250	5	2
Rheumatic Fever and Rheumatic Heart Disease	250	4	1.6
Diseases with hyperglobulinemia	80	4	5
Lupus erythematosus	20	1	5
Non-arthritic disease	560	17	3
Normal	200	2	1
Total	1380		

test are recorded in Table II.

TABLE II

RESULTS OF DEXTRAN PROCEDURE IN PATIENTS WITH RHEUMATOID ARTHRITIS

Clinical Diagnosis	No. of Patients	Positive No.	Tests %
Classic Rheumatoid Arthritis	15	15	100
Definite Rheumatoid Arthritis	182	181	99.4
Probable Rheumatoid Arthritis	26	21	80.7
Possible Rheumatoid Arthritis	23	15	65.2

The purpose of this research is to further compare the dextran method against two standard tests, the standard serum latex fixation and the euglobulin procedure with latex particles having been chosen.

PROCEDURE AND MATERIALS

Latex Fixation

Materials

1. Polystyrene particles of a uniform size of 81 micron, supplied by Difco as a suspension containing 11% solids.

2. Borate-saline buffer at pH 8.2. This is prepared by: (a) Add 50cc. of 0.1 M boric acid and 5.9 cc. of 0.1 N NaOH. Make up to 100 cc. with distilled HOH and adjust pH to exactly 8.2. (b) Add 0.85 gm. of NaCl to each 100 ml. of buffer solution.

3. Stock gamma globulin solution of 0.5% in borate buffer at pH 8.2. This is prepared by: (a) to 0.5 gm. of lyophilized fraction II add borate buffer in 5 and 10 ml. increments, mixing well and saving the supernatant until a total of 100 ml. has been added and the powder is completely dissolved. This can be stored for weeks under refrigeration.

4. Serum to be tested. It is not necessary for this serum to be inactivated or absorbed.

5. Isotonic saline.

Method

1. The first tube contains a serum dilution of 1:20 (0.1 cc. of serum and 1.9 cc. of buffer). A progressive twofold dilution of the serum being tested is prepared with borate-saline solution buffer at pH 8.2 so that each tube contains 1 ml. and a dilution from 1:20 to 1:5120.

2. Each test includes a control tube containing 1 ml. of buffered saline without added serum.

3. To each serum dilution tube containing one ml. and to the control tube is added 1 ml. of a mixture containing 1% stock latex and 0.5% stock gamma globulin in borate buffer. Note;, The latex-gamma globulin mixture is prepared by adding 0.1 ml. of stock latex and 0.5 ml. of stock 0.5% gamma globulin to 9.4 ml. of borate buffer. Ten ml. of this mixture is required for each test.

4. Shake the tubes thoroughly.

5. Incubate in a water bath at 56 degrees C. for two hours.

6. Centrifuge the tubes at 2300 rpm for three minutes.

7. Read the agglutination titer with the naked eye.

Agglutination in a titer of 1:20 or greater is positive.

Euglobulin Fractionation

Materials

1. 3 ml. of inactivated and absorbed serum. This is prepared as follows:

(a) Heat test sera at 57 degrees C. for 30 minutes;
(b) add an equal volume of packed sheep red cells for one hour at 37 degrees C.. Repeat (b) for a second absorption;
(c) test the absorbed sera against unsensitized cells to insure complete removal of the heterophil antibody.

2. 3 ml. distilled water.

3. M/150 citrate-phosphate buffer. This is prepared by:

(a) Add 100 ml. of 0.2 M Na_2HPO_4 and 75 ml. of 0.1 M citric acid. Dilute to 3 liters. Adjust the pH to 5.8 by the addition of small amounts of 1 N NaOH or the citric acid solution.

4. 3 ml. of 0.9% NaCl.

Method

1. 3 ml. of inactivated and absorbed serum are diluted with an equal volume of distilled HOH.

2. Dialyze in the cold at pH 6.0 against M/150 citrate-phosphate buffer for 48 hours with occasional agitation.

3. Wash the precipitate once with the buffer solution.

4. Dissolve the precipitate in 3 ml. of 0.9% NaCl.

Test the euglobulin thus obtained by the latex test as previously described.

Dextran Method for Detection of Abnormal Globulin Coating

Materials (not including those already on the list for the std. latex fixation test)

1. 3.6% dextran in physiologic saline. (This is prepared from 6% dextran in physiologic saline with an average molecular weight of 75,000 as available through Abbott Laboratories.)

2. Physiologic saline.

3. Blood from patient to be tested.

4. 10% sequestrene (EDTA).

Methods

1. 15 ml. of whole blood is collected from the patient and placed in test tubes containing 0.07 ml. of 10% sequestrene (EDTA).

2. Centrifuge at 2000 rpm for 10 minutes.

3. Separate 5 ml. of the packed red cells from the plasma and buffy coat and wash 3 times with physiologic saline.

4. The third saline washing is centrifugged at 14 degrees C. and 1600 rpm for 10 minutes.

5. Remove the supernatant, leaving the washed red cells.

6. Add 2.5 ml. of 3.6% dextran in physiologic saline; mix gently by inversion of the tube.

7. The solution is then allowed to stand at room temperature for 20 minutes and is then again centrifuged at 14 degrees C. and 1600 rpm for 10 minutes.

8. The supernatant is taken off and saved! (Supernatant #1)

9. The dextran-washed packed cells are then mixed thoroughly with 2.5 ml. of physiologic saline solution, allowed to stand at room temperature for 10 minutes and centrifuged at 14 degrees C. and 1600 rpm for 10 minutes.

10. The supernatant is again removed and saved. (Supernatant #2)

11. Test the supernatants saved in steps #8 and #10 by the latex-fixation test. A positive result is recorded with agglutination in either supernatant #1 or #2.

Sera were tested in three steps. First of all, the unknown serum and the dextran washings of the patient's erythrocytes were obtained. These materials were tested for their ability to agglutinate latex particles in the presence of gamma globulin. The euglobulin fraction of the unknown serum was obtained by dialysis against a dilute phosphate-citrate buffer at pH 5.8 and at 4 degrees C. for 48 hours. The euglobulin fraction of the unknown serum was then tested by the latex fixation test.

All three tests were by serial dilution technique. Agglutination was read in strong light against a dark background with the naked eye. A test was considered positive in dilution of 1:20 or higher.

The above three steps were used in testing sera

from 28 patients. The clinical diagnosis was not known in the laboratory at the time the sera were tested. After the tests were completed, diagnosis and laboratory data were evaluated. The results of this study are in Table III.

TABLE III

RESULTS OF COMPARISON STUDY OF SERUM LATEX, DEXTRAN, AND EUGLOBULIN PROCEDURES

Clinical Diagnosis	No. Cases	Serum Latex No.Pos.	%	Dextran No.Pos.	%	Euglobulin No.Pos.	%
Normal	2	0	0	0	0	0	0
Rheumatoid Arthritis	18	11	61.6	11	61.6	14	77.8
Osteoarthritis	3	0	0	0	0	0	0
Lupus Erythematosus	3	1	33.3	0	0	0	0
Fibromyositis	1	0	0	0	0	0	0
Rheumatoid Spondylitis & Psoriasis	1	0	0	0	0	0	0

DISCUSSION

Clinical diagnoses of the various disease entities were based upon standard criteria as stated in the Primer on the Rheumatic Diseases, 1959.

It should be pointed out that the patients with rheumatoid arthritis were in all stages of disease, some in acute exacerbation, some in complete remission, and

some who were suffering with chronic, long-standing disease. All had received a definite diagnosis of rheumatoid arthritis, as stated, and all were receiving or had received therapy for rheumatoid arthritis.

The results of the series of tests indicate, in contrast to previous reports, that the dextran procedure is not more sensitive than the serum latex procedure. It may be more specific than the serum latex procedure. In this series, the dextran test was positive in 61.6% of the rheumatoid arthritis patients tested. The standard latex fixation test was also positive in 61.6% of the rheumatoid patients tested. The dextran test was more specific in the sera of patients with lupus erythematosus. (See Table III.) No further differences between the standard latex test and the dextran test were noted.

It should be stated that two of the patients with rheumatoid arthritis who were in the series also had associated syndromes of a marked Raynaud's phenomenon and a lymphomatous disease. The results did not differ as far as the serologic tests were concerned.

It was noted that the most sensitive and specific test was that utilizing latex particles and the euglobulin fraction. In the rheumatoid sera tested, in all stages of the disease, this test was positive in 77.8% of all cases. These results are similar to previous series which

did not include the dextran procedure. The euglobulin-latex procedure has consistently been reported as the most sensitive test for the rheumatoid factor with the exception of the euglobulin inhibition procedure (not included in this series). The increased sensitivity utilizing the euglobulin-latex procedure is certainly sufficient, in this study, to be significant.

Three sera, from patients in the University of Nebraska hospital, were cross-checked by the serial tube dilution techniques (as used in this series) and the macro-agglutination slide technique as utilized in the hospital serology laboratory. One of the three sera tested was reported negative by the hospital laboratory. In serial tube dilution technique, on the same serum, a prozone phenomenon was noted in that 1:20 and 1:40 dilutions were negative but all higher dilutions through 1:640 were positive.

The prozone phenomenon has been reported in other series, most recently in a review by Singer (13). This phenomenon has been described as due to a thermolabile substance with the characteristics of serum complement or to a gamma globulin inhibitor. The phenomenon is undoubtedly due in part to the non-specific role of high concentrations of other serum proteins in the test system.

Laboratories which utilize a macro-agglutination

slide procedure in serological diagnosis should definitely be aware of the hazard of the prozone phenomenon in their technique. Serial tube dilution techniques are more efficient in dealing with the prozone phenomenon.

As a result of these studies, it is our opinion that the dextran procedure is neither practical nor feasible for the majority of hospital laboratories. Not many laboratories are equipped with refrigerated ultracentrifugation. The procedure is slightly more expensive, although this is a minimal factor, and requires considerably more time. As has been stated, it did not show increased sensitivity in this study.

Serologic tests are a definite laboratory aid in diagnosis of rheumatoid arthritis. However, serologic tests detect macroglobulins collectively referred to as "rheumatoid factors." These factors are present in many other disease entities, thus causing many false positive reactions. In general, high titers are encountered in active rheumatoids, but low titers frequently occur even in the presence of clinical activity. This adds more discrepancy to the reliability of the tests. A positive serologic test must therefore be considered as only an aid and must be correlated with clinical, pathologic and roentgenologic changes. A negative test does not rule out rheumatoid arthritis.

SUMMARY

A study of the sera of 28 patients with various disease entities revealed:

1. No increase in sensitivity by use of the dextran procedure as compared to the use of the serum latex procedure.
2. Greatest sensitivity by use of the euglobulin-latex procedure.
3. The dextran procedure is not a practical procedure for most diagnostic laboratories.
4. Serology studies for the "rheumatoid factor" should be used merely as an aid in diagnosis of rheumatoid arthritis and must be correlated with clinical, pathologic and roentgenologic data.

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